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Nitric oxide and interleukin 8 production by the human colon

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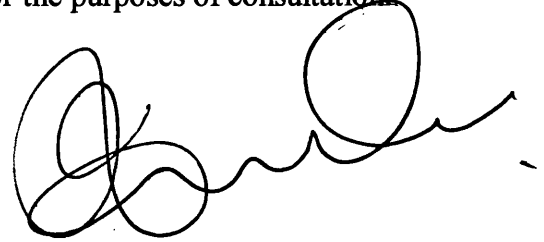

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Nitric Oxide And Interleukin 8 Production By The Human Colon

Summary

The aetiology of inflammatory bowel disease is unknown. Previous work has shown that nitric oxide (NO) is produced in high concentrations in the human colon when inflammation is present in ulcerative colitis, and that NO concentrations diminish when the inflammation resolves. The enzyme responsible for this is believed to be the inducible form of nitric oxide synthase (iNOS). In human colonic mucosa iNOS expression is limited to epithelial cells and previous work on colonic epithelial cell lines e.g. HT-29 cells demonstrates that expression can be induced by stimulation using a mixture of pro-inflammatory cytokines (IL-1, TNF-α and IFN-γ). Expression can be modulated by the T-cell derived cytokines IL-4 and IL-13 but is not affected by IL-10 despite the evidence that IL-10 has some effect in clinical practice.

The work described in this thesis shows that two steroids, prednisolone and budesonide, commonly used in the treatment of colitis have no effect on either NO or interleukin-8 production by stimulated HT-29 cells in clinically relevant doses. Further work on colonic biopsies from patients with newly diagnosed colitis showed that they produced NO and IL-8 in large quantities and that this could be inhibited with small concentrations of either prednisolone or budesonide. iNOS and IL-8 mRNA expression in these biopsies were measured using a semi-quantitative polymerase chain reaction (PCR) technique which showed a significant fall in levels when the biopsies were treated with either steroid. Work on the effect of the three T-cell derived cytokines on the colonic biopsies showed that the production of both NO and IL-8 was easily inhibited by both IL-4 and IL-10. Expression of iNOS and IL-8 mRNA was also inhibited by IL-4 and IL-10. The effect of IL-13 was much less pronounced.

Colonic biopsies from histologically normal colon in patients with and without colitis were stimulated with the same pro-inflammatory cytokine mix that was used on the HT-29 cells. A significant increase in NO production and IL-8 levels and a rise in iNOS mRNA and IL-8 mRNA was demonstrated. Increased IL-8 expression was inhibited by the T cell derived cytokines in histologically normal biopsies from patients with or without colitis but NO production and iNOS mRNA expression was only inhibited in histologically normal biopsies from patients with colitis.

To examine further why colonic biopsies and HT-29 cells respond differently to T-cell derived cytokines and steroids with respect to NO and IL-8 production, the effect of these substances was studied in a co-culture of peripheral blood mixed mononuclear cells (MMCs) and HT-29 cells. The co-culture produced large quantities of NO when stimulated with IFN-γ and lipopolysaccharide (LPS), but there was no effect on NO production in the HT-29 cells or MMCs when cultured alone. Conditioned media from the LPS and IFN-γ treated MMCs induced NO production in HT-29 cells, demonstrating that the MMCs produced a soluble factor or factors that stimulate NO production in the HT-29 cells. This effect was inhibited by treatment of the HT-29 cells with IL-1 receptor antagonist or by treatment of the MMCs with prednisolone or budesonide or all of the T cell derived cytokines including IL-10. The inflammatory responses of epithelial cells in intact colonic mucosa appear to be largely determined by their interaction with other cell populations within the mucosa such as MMCs.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
5-ASA	5-Aminosalysilate
ATP	Adenosine triphosphate
BH_4	Tetrahydropterin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-5'-monophosphate
CD	Crohn's disease
cNOS	Constitutive nitric oxide synthase
Con-A	Conconavalin
CSF-1	Colony-stimulating factor-1
DAB	3,3' Diaminobenzidine
DAN	2,3-Diaminonaphthalene
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ENA-78	Epithelia-derived neutrophil attractant-78
FCS	Foetal calf serum
GCP-2	Granulocyte chemotactic protein-2
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HLA	Human leukocyte antigen
HLA-DR	D-related HLA
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon-γ

IGF-1	Insulin-like growth factor-1
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
IP-10	IFN-γ-inducible 10
LFA-1	Leukocyte function antigen-1
L-NMMA	N^{ω}-monomethyl-L-arginine
LPS	Lipopolysaccharide
LT	Leukotriene
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1α
MOPS	3-[N-morpholino]propane-sulphonic acid
mRNA	Messenger ribonucleic acid
MUC	Mucin
NAD	Nicotinamide adenine dinocleotide
NADPH	Nicotinamide adenine dinocleotide phosphate
NAP-2	Neutrophil activating peptide-2
NF-κB	Nuclear factor- κB
NO	Nitric oxide
NOS	Nitric oxide synthase(s)
NSAID	Non-steroid anti-inflammatory drug
OD	Optical density
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
RANTES	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species

SC	Secretory component
SCFA	Short chain fatty acids
SDF-1α	Stromal cell-derived factors 1α
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIgA	Secretory IgA
SOD	Superoxide dismutase
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	N, N, N', N'-tetramethylethylene diamine
TGFβ	Transforming growth factor-β
TNF	Tumour necrosis factor
Tween-20	Polyoxyethylenesorbitan monolaurate
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
UC	Ulcerative colitis
VLA_4	Very late antigen-4

Chapter 1

INTRODUCTION

1.1 Inflammatory Bowel Disease

1.1.1 Historical perspective

There are two idiopathic inflammatory bowel diseases (IBD): Ulcerative Colitis (UC) and Crohn's Disease (CD). Although originally thought to be two ends of a spectrum of a single disease, it has become clear that they are two different diseases and thus should be considered separately. Both CD and UC are thought by some to be 20th Century diseases, however, they were probably originally described some time before this.

Morson's review of Matthew Baillies 1793 *Morbid Anatomy of Some of the Most Important Parts of the Human Body* shows that patients were dying from ulcerating conditions of the colon during the later part of the 18th Century (Morson, 1970). Crohn's disease was probably described as long ago as 1813 by Combe and Saunders who described a case of stricturing of the lower part of the ileum as far as the colon that was "constricted for the space of three feet to the size of a turkey quill" (Combe and Saunders, 1813). In 1828 Abercrombie reported some further cases of a regional ileitis (Abercrombie, 1828). In 1889 a further patient with what would probably become known as Crohn's Disease was described by Fenwick in a series of lectures given in London (Fenwick, 1889). Ulcerative Colitis was probably first described in 1859 by Samuel Wilks, a physician at Guy's Hospital, who recognised that "simple, idiopathic colitis"; could be distinguished from other forms of colitis, such as bacterial dysentery.

This original case concerned a 42 year old woman who died after several months of diarrhoea and fever. An autopsy demonstrated a transmural inflammation of the colon thought to be unlike other infectious causes known at the time (Wilks, 1859). By 1907 over 300 patients with inflammatory changes of the bowel had been reported in a survey of seven London hospitals. Nearly one-half of the patients died from perforation of the colon with peritonitis (Cameron and Rippman, 1909). In 1907 the increased incidence of colonic carcinoma in those patients with ulcerative colitis was recognised by Lockhart-Mummery (Lockhart-Mummery, 1907). Speculation as to the cause of colitis began to increase and in 1921 Hurst implicated an organism "related to *B. dysenteriae*" (Hurst, 1921). A daily treatment regimen of the colon with dilute solutions of silver nitrate or tannic acid and the intravenous administration of an anti-dysenteric serum was tried with little effect.

Further descriptions of what in future would be known as Crohn's disease, was described by Dalziel in 1913 in the British Medical Journal. This description was of a condition causing a chronic interstitial enteritis (Dalziel, 1913). Crohn described a detailed description of several cases of regional ileitis in a series of articles from 1932 (Crohn *et al.*, 1932;Crohn, 1934;Crohn, 1949)

Between 1930 and 1950s sulphanilamide and penicillin were tried with increasing success. A major breakthrough came with the use of ACTH and adrenal steroids which were introduced for treatment in 1950. In 1951 the complete reversibility of ulcerative colitis was demonstrated in a group of patients who responded to steroid therapy (Kirsner *et al.*, 1951). Because of this success with steroids, recent developments in therapeutics and searches for aetiologies of IBD have concentrated upon the premise that IBD is an inflammatory condition rather than

having an infective aetiology. Although the overall prognosis of ulcerative colitis and Crohn's disease has improved over the years, a cure has still not been found. Until the causes of these diseases have been clearly identified, therapeutic strategies for these conditions continues to be based on interrupting the immunological mechanisms involved and inhibiting the gut inflammation.

1.1.2. Pathological features of IBD

The inflammation in UC is superficial, that is, it is limited to the mucosa and submucosa, and is confined to the large bowel. It affects the rectum and a variable length of the colon proximally. Histologically, the disease is characterised by an infiltration of both acute and chronic inflammatory cells in the lamina propria and the crypts which form microabscesses. The deeper layers of the intestinal wall are characteristically not involved.

In contrast, CD is a transmural inflammation that may occur anywhere throughout the alimentary tract from the mouth to the anus. The disease can affect the deeper layers of the bowel wall producing superficial and deep ulceration and can also leading to collagen deposition and fibrosis. The presence of granulomata is a characteristic feature of Crohn's disease.

Both diseases are characterised by a chronic relapsing course and associated with many extra-intestinal manifestations. Increasing evidence suggests that the two conditions are distinct although they share important common pathological mechanisms. It seems apparent that the tissue injury seems to be mediated by the immune system, although whether this is the initiating event or a secondary event is unclear. It is likely that the aetiology of IBD is not a single cause and probably represents an interaction between various factors both host and environmental. In

the following section, recent clues in aetiology and pathogeneses of IBD are reviewed, concentrating mostly on immunological mechanisms.

1.2 Aetiological Factors in IBD

Although it seems likely that different external aetiological factors act upon a genetically susceptible individual, this is by no means proven. A number of genetic influences, especially immunological abnormalities (host), dietary and infectious agents (environmental) have been suggested. These are discussed in more detail below.

1.2.1.Genetics.

The importance of genetic factors is suggested by clustering of cases within families and an association amongst different racial and ethnic groups. The frequency of IBD in first-degree relatives varies between studies but can be as high as 40% (Farmer and Michener, 1986). Patients with CD have a stronger family history than patients with UC although there is also a strong possibility of patients with Crohn's disease having a first degree relative with UC and *vice versa*. Further evidence of a strong genetic influence in inflammatory bowel disease has come from twin studies with increased concordance of both UC and CD in monozygotic compared with dizygotic twins. The concordance rate of monozygotic twins with CD is higher than that of twins with UC (Tysk *et al.*, 1988). These studies suggest the existence of a polygenic genetic disease in the aetiology of IBD rather than a simple Mendelian pattern of inheritance. The search for specific genetic markers has so far been unrewarding. Diseases such as ankylosing spondylitis and sclerosing cholangitis, which occur in association with

IBD, have strong associations with particular human leukocyte antigen (HLA) antigens thus suggesting indirect evidence for a genetic relation of these antigens with IBD. HLA class II antigens have been studied in depth with differing results between different population groups, e.g. there is a strong and reproducible association of HLA-DR2 in Japanese patients with UC, but no association is observed in patients with UC from the UK or the USA. Instead in this group association with HLA-DRB1*0103 and DRB1*1502 has been noted (Asakura *et al.*, 1982; Satsangi *et al.*, 1996; Duerr and Neigut, 1995). Association with specific genetic markers such as for anti-neutrophil-cytoplasmic-autoantibodies (ANCA) or for specific cytokine abnormalities e.g. TNFα and IL-1ra polymorphisms have been sought although the relationship is generally very weak (Duerr and Neigut, 1995; Mansfield *et al.*, 1994; Yang *et al.*, 1995). A strong association between a particular locus on chromosome 16, the so called IBD1 locus and Crohn's disease and a locus on Chromosome 12, the IBD2 locus and UC have been shown in different studies (Mirza *et al.*, 1998; Hamlin *et al.*, 1999). Recently, it has been shown that a particular TNF-α allelic combination is a very strong genetic risk factor for CD and with HLA class II alleles, defines a group of markers on chromosome 6 that extends from HLA class II to upstream of the TNF-alpha gene (Plevy *et al.*, 1996; Hampe *et al.*, 1999).

1.2.2. Mucus.

Mucus represents a major component of intestinal defence and defects of this layer could allow the development of IBD. The mucus produced by the colon in patients with UC and CD is qualitatively different from that produced by normal subjects, reflecting a change in the structure of colonic mucin (Saitoh *et al.*,

1996). Mucin is a heavily sulphated glycoprotein and it has been shown that enzymatic desulphation by faecal bacterial sulphatases greatly increases the susceptibility of the mucin to degradation. The increased faecal mucin sulphatase activity in UC could be the result of greater quantities of mucin leading to bacterial enzyme induction, but this would probably result in more rapid degradation of secreted mucin and represents a potential target for treatment (Tsai *et al.*, 1995). Compared with healthy subjects, the different biochemical features of the mucin obtained from whole gut lavage fluid from IBD patients appeared to reflect mucosal pathological changes associated with the disease (Saitoh *et al.*, 1996). It is unclear whether this is secondary to any inflammation present, or whether this is a primary deficit which may predispose patients to acquiring UC and CD. The oligosaccharide side chains of mucus glycoproteins are shorter in patients with UC than controls, and bind differently with monoclonal antibodies and lectins (Rhodes *et al.*, 1988; Podolsky and Fournier, 1988). The predominant mucin in the human colon responsible for the protective mucus layer is MUC2. Precursor and total MUC2 levels were significantly decreased in UC patients with active inflammation compared to controls, and returned to control values during remission of the inflammation (Van Klinken *et al.*, 1999). The marked increase in mucin synthesis that results when butyrate is added to the media in colonic biopsy cultures, suggests that butyrate may be an important mechanism affecting the rate of mucin synthesis *in vivo* and may also explain the therapeutic effect of butyrate in colitis (Finnie *et al.*, 1995). A similar rise in mucin synthesis with treatment with corticosteroids may explain in part, the therapeutic effect of steroids in UC (Finnie *et al.*, 1996)

1.2.3 Mucosal Permeability.

It has been found that when the colon becomes inflamed, it becomes more permeable and thus absorbs usually impermeable substances such as lactulose and Cr-EDTA. Significant increases in blood concentrations of these molecules in patients with IBD have been found compared with controls, suggesting an increase in intestinal permeability in patients with IBD. This disturbance of the intestinal barrier may be due to changes in the mucosa such as erosion, ulceration, and oedema (Hollander, 1992; Bjarnason *et al.*, 1995). Determining whether healthy first-degree relatives of patients with CD have increased permeability is of considerable importance because it could represent a primary predisposing factor which can lead to inflammation and clinical disease later in life. It has been discovered that increased permeability can antedate clinical manifestations of CD (Wyatt *et al.*, 1993). It has been suggested that this increase in permeability may be genetic in origin. Lindberg et al using monozygotic twin pairs both concordant and discordant for CD, found no differences in the absorption of polyethylene glycols between the study groups (Lindberg *et al.*, 1995). Although permeability is normal in most relatives, it has been found that a subgroup of patients had markedly abnormal permeability suggesting that they may represent those most at risk of developing CD (May *et al.*, 1993). Another study from the same group, found that when relatives are challenged with acetylsalicylic acid, permeability increases in some of them (Hildsen *et al.*, 1996). The possible consequences of abnormal permeability have been shown by the fact that relatives have an increase in the number of circulating $CD45RO^{+}$ B cells (Yacyshyn and Meddings, 1995). This suggests that a leaky intestinal barrier allows increased antigen absorption, which in turn, leads to an exaggerated immune stimulation reflected at a systemic

level by increased numbers of B cells. These results give some support to the hypothesis of a genetically determined intestinal "leakiness" in CD and how this may link in to the well-documented immune abnormalities. Whether increased gut permeability in CD is a primary abnormality predisposing to increased mucosal access of luminal pathogenic factors, or is simply a consequence of mucosal damage induced by other mechanisms is not yet resolved.

1.2.4 Colonocyte Energy Metabolism.

It has become evident that in the normal colonic epithelium the major source of energy are short chain fatty acids (SCFA). The principal SCFAs are acetate, propionate, and butyrate, which are normally formed by bacterial fermentation of unabsorbed carbohydrates (Cummings and Macfarlane, 1991). These are present in colonic contents in relatively constant concentrations and proportions between different people. They are absorbed from the colonic lumen, and once inside the colonocytes the SCFAs undergo oxidation. In UC, colonocytes have been shown to utilise butyrate as an energy source less efficiently and it is postulated that low luminal SCFA levels in severe UC may play a part in the disease process (Roediger, 1980). Thus correction of these low levels may help in the treatment and so clinical trials of SCFA enemas have been performed. These have been shown to have a therapeutic benefit in patients with distal UC (Breuer *et al.*, 1991) Another trial using Plantago ovata seeds, which when they undergo colonic fermentation yield butyrate, showed they were as effective as mesalazine in maintaining remission in ulcerative colitis (Fernandez-Banares *et al.*, 1999). This suggests that UC could be an expression of an energy deficiency disease of the colonic mucosa. Whether abnormal colonic epithelial SCFA utilisation in UC is a

primary or secondary phenomenon, perhaps due to altered bacterial flora, is currently unclear.

1.2.5.Diet.

Because the main role of the gastrointestinal tract is to process and absorb food, it is reasonable to propose that dietary factors, possibly acting as antigens, play a role in the initiation or persistence of intestinal inflammation. There is less evidence to support this hypothesis in UC than in CD, although even in CD the role of dietary antigens in the aetiology of is controversial. In patients with CD, elemental diets i.e. where complex food substances have been chemically broken down into their constituent parts, appear to have a therapeutic effect, although the mechanism by which this occurs is uncertain. Controlled studies have suggested that elemental diets may be as effective as corticosteroids in inducing a remission in patients with acute CD (O'Morain *et al.*, 1984). Favourable reports of diets, both exclusion diets (Riordan *et al.*, 1993), or specific diets containing substances such as fish oils, indigestible oligosaccharides, gum arabic and antioxidants (Campbell *et al.*, 1997) have been described by some groups but not by others.

Population-based case-control studies of IBD especially examining dietary habits has shown patients with CD were more likely to have a diet featuring a high sucrose intake and with less intake of fibre (Kasper and Sommer, 1979; Persson *et al.*, 1992). UC patients have been shown to consume more protein than controls (Tragnone *et al.*, 1995).

Dietary therapies have included an enteric-coated preparation of fish oil that has been shown to be effective in patients with CD (Kim, 1996). In UC, avoidance of dairy products has shown an improvement in a minority of patients, but

intravenous feeding, which because it is removing all antigenic stimuli from the GI tract would be expected to prove beneficial, was not found to be effective and in some cases even made the disease worse (Burke *et al.*, 1997).

1.2.6. Infection.

Crohn's disease has some parallels with other known infectious diseases e.g. Yersinnia, so work has been done on examining the possible role of an infectious agent in the aetiology of IBD. Pathological resemblances between Johne's disease in cattle, which is caused by Mycobacterium paratuberculosis, and CD have resulted in study of the possible aetiological role of this organism (Chiodini *et al.*, 1984; Sanderson *et al.*, 1992). Immunohistochemical studies have generally failed to show the organism, however studies using the polymerase chain reaction (PCR) have detected DNA from M. paratuberculosis in CD affected bowel much more often than in UC or in control bowel. However data from other studies such as using anti-tuberculous therapy does not support a role for M. paratuberculosis in Crohn's disease (Kobayashi *et al.*, 1988; Kobayashi *et al.*, 1989). Multiple studies from one group has suggested that measles or another paromyxovirus is important in the aetiology of Crohn's disease because of the possible identification of the virus in the vascular endothelium of the intestine in CD (Wakefield *et al.*, 1993; Wakefield *et al.*, 1995; Wakefield *et al.*, 1997). There is some epidemiological data supporting the idea that early infection with the measles virus either in childhood or prenatal exposure is a risk factor for the later development of CD (Ekbom *et al.*, 1994). This data is consistent with the possibility that CD may be a chronic granulomatous vasculitis reaction to a persistent infection within the vascular endothelium (Wakefield *et al.*, 1989; Wakefield *et al.*, 1997).

It would appear that the balance between the protection given by mucosal forces and luminal microbial contents can be disturbed by changes in the environment or genetically determined abnormalities in the immune system could lead to a chronic, relapsing intestinal inflammation (Sartor, 1997). It would seem unlikely that the change in the environment could be due to an infection with a single agent. Another explanation is that more than one infectious agent can trigger IBD, possibly by stimulation of a susceptible potentially over-responsive immune system (Macpherson *et al.*, 1996).

1.2.7.Cigarette smoking.

As UC occurs primarily in non-smokers and ex-smokers it appears that smoking is somehow protective against the onset of disease (Harries *et al.*, 1982). This is in contrast to the disease course of patients with CD, which is less favourable in smokers than in non-smokers (Somerville *et al.*, 1984; Timmer *et al.*, 1998). Nicotine whether in chewing gum, transdermal patches or topically applied have all been shown to improve clinical symptoms and endoscopic appearance in patients with active UC (Pullan *et al.*, 1994; Dash *et al.*, 1999; Sandborn, 1999; Green *et al.*, 1997). To date no maintenance benefit has been demonstrated. Suggested mechanisms for protective effects of nicotine in ulcerative colitis are many and varied including direct effects on mucosal defences and physical properties of the mucosa e.g. increased mucus production (Zijlstra *et al.*, 1994; Cope and Heatley, 1992), increased release of endogenous glucocorticoids by epithelial cells, (Cope and Heatley, 1992), alteration of rectal mucosal blood flow, decreased intestinal permeability, and effects on the immune response e.g. decreased mucosal eicosanoid levels (Zijlstra *et al.*, 1994), release of endogenous

glucocorticoids, significant inhibition of Interleukin-2 (IL-2) and TNF-alpha production (Madretsma *et al.*, 1996a; Madretsma *et al.*, 1996b; van Dijk *et al.*, 1998) and an inhibition of IL-10 production (Madretsma *et al.*, 1996b). This possibly explains the detrimental effects of smoking and nicotine in CD, although does not explain why UC and CD behave so differently with relation to smoking.

1.2.8.Drugs

As the incidence of colitis seems to have risen dramatically in the 20th century, one possible explanation for this is the increased use of various drugs and medications. One of the most likely candidates is the group known as non-steroidal anti-inflammatory drugs (NSAIDs). These have been associated with initiation and relapse of IBD, possibly due to changes in eicosanoid metabolism and gut permeability (Rampton, 1987).

There is conflicting evidence of an increased risk of CD in users of the oral contraceptive pill (Boyko *et al.*, 1994; Lesko *et al.*, 1985; Cosnes *et al.*, 1999; Timmer *et al.*, 1998; Lashner *et al.*, 1989), with the prothrombotic effects of the drug perhaps explaining any increase in the likelihood of a worsening of Crohn's Disease. A theoretical disease model has been described with intestinal ischaemia caused by thromboembolic events in small blood vessels supplying the mucosa, which trigger a chain of events that culminate in clinical IBD (Koutroubakis *et al.*, 1996), although most of these studies it is hard to isolate the effect of the oral contraceptive pill whilst not including the effect of smoking.

There are some individual reports relating antibiotic usage to relapse of IBD and a possible explanation for this is due to changes in colonic bacterial flora although

some authors paradoxically advocate the use of antibiotics in severe colitis (Peppercorn, 1993).

1.3.The Role Of Colonic Epithelium In The Immunology Of The Bowel

1.3.1.Epithelial Cell

The colonic mucosa is the layer of the large bowel which divides the luminal contents of the colon from the large bowel structure. It forms the absorptive surface and covers the lamina propria, which is found immediately beneath the epithelium and the crypts. The main cell populations in the colonic epithelium are the colonic epithelial cells and mucus secreting goblet cells. Intraepithelial lymphocytes infiltrate throughout the epithelium. The primary function of the intestinal epithelium is the absorption of nutrients, water and electrolytes from the gut lumen into the body. The colonic epithelial cell was originally thought of as a barrier, with a role in transport of nutrients, but probably immunologically inert. However, during the last two decades it has been realised that the epithelium itself is instrumental in the inflammatory process.

The evidence that the epithelial cell could be involved in the immunological response of the mucosa first appeared in 1970 with the discovery of IgA antibodies. This demonstrated that the function of IgA is dependant on epithelial cells. It was shown that mucosal IgA is synthesized by plasma cells as a dimer. This dimer then combines with a transmembrane polypeptide produced by epithelial cells, called secretory component, to form secretory IgA (Tomasi, Jr., 1970). This peptide protects the IgA dimer from luminal proteolysis (Mostov *et al.*, 1984). It was also reported that IgM function at mucosal surfaces was in the

form of secretory IgM, where IgM binds to secretory component (Brandtzaeg, 1973).

The primary function of the intestinal epithelial cells is to act as a barrier between the environment and the host. As the epithelial cells are also the first cells to come in contact with many pathogens, it is likely that they play a critical role in alerting the immune system located in the underlying intestinal mucosa. These in turn are involved in the recruitment of circulating immune cells during the inflammatory response.

Within the last decade colonic epithelial cells have been increasingly recognised as having an active role in inflammation, with roles such as presenting antigens via class II MHC expression and thus acting as antigen presenting cells to T cells (Mayer *et al.*, 1991; Lowes *et al.*, 1992). Although epithelial cells in the normal small intestine express MHC Class II determinants, only epithelial cells in the inflamed colon do so (Selby *et al.*, 1983) Colonic epithelial cells also express adhesion molecules e.g. intracellular adhesion molecule-1 (ICAM-1) (Kvale and Brandtzaeg, 1995) and can produce soluble inflammatory mediators e.g. arachidonic acid derivatives (Dias *et al.*, 1992), platelet activating factor (Ferraris *et al.*, 1993), cytokines (Hedges *et al.*, 1992; Eckmann *et al.*, 1993; Jung *et al.*, 1995; Schuerer-Maly *et al.*, 1994; Gross *et al.*, 1995), and chemokines (Mazzucchelli *et al.*, 1994; Eckmann *et al.*, 1994), all of which allow communication between cells of the immune system. Colonic epithelial cells appear to provide a set of signals to allow and promote the activation of the inflammatory response in the early phases of attack from bacteria etc.

Despite the obvious proximity of various populations of white cells, e.g. macrophages, lymphocytes to the epithelial cells in the mucosa, little if any work

has been done on the relationship between these two cell populations. Almost all work concentrates on isolated groups of cells e.g. epithelial cells on their own. It is very likely however, that when the local macrophage and lymphocyte populations are stimulated by antigens, they in turn then influence the epithelial cells. This in turn will lead to mediators being released from the white cells and acting locally on the epithelial cells. No information about which mediators are important or even what happens when the two populations of cells are in contact is available.

1.3.2 Intra-Epithelial Lymphocytes

The exact role of intra-epithelial lymphocytes (IEL) in the gastrointestinal mucosa is poorly understood (Croitoru and Ernst, 1992; Cerf-Bensussan and Guy-Grand, 1991). It is thought that IEL play a central role in local intestinal immunity and are likely to be important in defence against gastrointestinal neoplasms. There are two populations of IELs; the predominant group is the $CD8^+$ (suppressor-cytolytic) subset of T lymphocytes that display the α/β T-cell receptor (TCR) and show evidence of activation. The second group of T cells are mostly $CD4^-$ and bears the γ/δ form of TCR. The majority of the lamina propria T lymphocytes belong to the α/β form of T cells with the minority being γ/δ cells (Trejdosiewicz, 1992).

Animal models of inflammatory bowel disease have shown that a chronic inflammatory bowel disease occur spontaneously in mouse models where targeted deletions of immune response genes have taken place. These so called knockout" mice models are able to develop colitis with a wide range of products deleted e.g. IL-2(Sadlack *et al.*, 1993), IL-10 (Kuhn *et al.*, 1993), T cell receptor (Mombaerts

et al., 1993), TGF-β (Kulkarni *et al.*, 1993). Recently important studies using similar animal models have demonstrated that when T lymphocytes are transferred into T-cell deficient mice then colitis will occur (Rudolphi *et al.*, 1994). It would appear from experiments using these models that most events in the inflammatory response both pro-inflammatory and regulatory are controlled by $CD4^+$ T lymphocytes (Elson *et al.*, 1995;Elson *et al.*, 1995). If these knockout mice are kept in sterile environments then colitis does not occur. This would suggest that the mucosal inflammation is antigen dependant and the responsible antigens originate from intestinal bacteria (Schultz *et al.*, 1999; Sellon *et al.*, 1998). These studies show the key role of different parts of the immune response and do suggest an important role of T cells especially intraepithelial lymphocytes in the pathogenesis of the immune response.

1.4.Immunological Factors In IBD

It is known that IBD involves a widespreal activation of the immune system and many of the effects of inflammatory bowel disease are caused by the excessive and tissue damaging chronic inflammatory response in the gut wall. Down-regulating this response allows the mucosa to heal and function of the gastrointestinal tract to return to normal. Corticosteroids are extremely good at inhibiting immune responses and in the majority of patients are the mainstays of treatment. However they merely control the inflammation and do not permanently alter the natural history of IBD.

The inflammatory reaction involves early expression of surface adhesion molecules on vascular endothelial cells, with consequent migration and subsequent activation of circulating leukocytes into the colonic mucosa

(Schuermann *et al.*, 1993; Koizumi *et al.*, 1992). This activation is attributed to the synthesis of inflammatory mediators and cytokines that in turn contribute to the pathological features of IBD. These mediators are produced not only by both resident and newly recruited mucosal inflammatory cells such as neutrophils, macrophages and mast cells, but also by colonic epithelial cells (MacDonald and Murch, 1994).

1.5. Cytokines and Inflammatory Bowel Disease

Cytokines are glycosylated peptides ranging in size from 6 to 30 kilodaltons secreted by a wide variety of cells in response to immunological challenge, such as infection, inflammation or tissue injury. Very small quantities are known to have powerful biological effects. Even in picomolar concentrations cytokines elicit numerous responses from target cells. They are therefore important in all aspects of the regulation of the immune response. They are involved in both the initiation and the amplification of the immune response (MacDermott and Stenson, 1988). Cytokines can be sub-divided into the following different families; Interleukins (e.g. Interleukin-1), Interferons (e.g. Interferon-α, Interferon-γ), Tumour necrosis factor family (e.g. TNF–α), Colony Stimulating factors (e.g. G-CSF, EPO), Chemokines (e.g. IL-8 GRO–α, ENA-78, MIP-1α, MCP-1, RANTES, Eotaxin) and Growth Factors (e.g. TGF-β1, TGF-α). Some cytokines are thought to have mainly pro-inflammatory effects for example, Interleukin 1 (IL-1), Interleukin 8 (IL-8), and some are found to have mainly anti-inflammatory actions e.g. Interleukin 10 (IL-10).

1.5.1 Interleukin -1

IL-1 was one of the first cytokines to be identified in experimental animals and humans. It was found that a soluble factor was able to cause fever and was thus initially described as "endogenous pyrogen" (Atkins, 1960). It was soon found that in addition to causing fever, it could mediate many other functions. These include prominent pro-inflammatory effects, for example, induction of acute phase proteins, neutrophilia and an increase in synthesis of serum amyloid A protein (Dinarello, 1984). It has also been known by a wide variety of names including leukocyte endogenous mediator, lymphocyte-activating factor, mononuclear cell factor, catabolin, osteoclast-activating factor and haemopoietin (Dinarello, 1989).

Although IL-1 can be produced by a wide variety of both immune and non-immune cell types, most IL-1 is produced by monocytes and macrophages. Two isoforms of the protein have been identified although both use the same cell surface receptor. IL-1α is mainly membrane associated and IL-1β is mainly secreted. Both isoforms are able to modulate the functions listed above and also have other major effects. These include acting upon endothelial cells leading to the accumulation of both platelets and leukocytes, stimulation of collagenase production, stimulation of fibrogenesis, increased production of prostaglandins and leukotrienes, and activation of mature T and B cells (Dinarello, 1989). Raised levels of IL-1 are found in a wide variety of pathological conditions including septic shock, rheumatoid arthritis, acute and chronic myeloid leukaemia and inflammatory bowel diseases.

Organ cultures of inflamed mucosa from patients with IBD were found to produce increased amounts of IL-1β (Reimund *et al.*, 1996). Increased concentrations of

IL-1 have been reported in active ulcerative colitis although the exact site of synthesis remains unclear (Ligumsky *et al.*, 1990). IL-1 production in stimulated peripheral blood mononuclear cells from patients with active ulcerative colitis and Crohn's disease was significantly increased compared to controls. The levels correlated with serum C-reactive protein. Furthermore when patients were in clinical remission IL-1 levels fell to normal (Nakamura *et al*, 1992; Mazlam and Hodgson, 1992). When looking at mononuclear cells from the intestinal lamina propria from patients with active ulcerative colitis and Crohn's disease again increased levels of IL-1β were found. These findings were confirmed when looking at the supernatant of colonic mucosal biopsies from patients with active inflammatory bowel disease (Mahida *et al.*, 1989).

It has been shown that pre-treatment of rabbits with a small dose of IL-1 reduces subsequent inflammation in an immune complex model of inflammatory bowel disease. This is very similar to the effect seen in shock induced by endotoxin. The precise mechanism of this is unknown but it may be that a small dose of IL-1 induces gene transcription of a protective factor or factors that result in the down regulation of inflammation (Cominelli, 1990).

1.5.2 Interleukin-1 Receptor Antagonist

In 1990 a new peptide was purified, cloned and expressed which is a natural antagonist of IL-1 and has been named Interleukin-1 receptor antagonist (IL-1ra)(Eisenberg *et al.*, 1990). This specifically blocks IL-1 activity and not other pro-inflammatory mediators. Two structural variants have been described; a secreted form primarily produced by mononuclear cells (Eisenberg *et al.*, 1990) and an intracellular form found in epithelial cells (Haskill *et al.*, 1991). Elevated

plasma and tissue levels have been detected in various inflammatory diseases and it has been suggested that it may be part of the homeostatic mechanism designed to down regulate the immune response (Dinarello and Wolff, 1993). It has been detected in normal and inflammatory bowel disease affected tissue (Isaacs *et al.*, 1992). The likely sources of IL-1ra are lamina propria mononuclear cells and epithelial cells (Bocker *et al* 1998). It has been suggested that an imbalance in the relative proportions of IL-1 and IL-1ra could result in an inability to produce an appropriate inflammatory reaction.

1.5.3.Tumour Necrosis Factor-α

Two major forms of tumour necrosis factor exist, tumour necrosis factor-α (TNF-α or cachectin) and tumour necrosis factor-β (TNF-β or lymphotoxin). They are both key inflammatory cytokines that share several properties with IL-1. TNF-α was initially described in the peripheral circulation of animals with severe bacterial infection. It was found to be able to induce tumour destruction (Carswell, 1975). This cytokine is derived primarily from macrophages and monocytes, although lymphocytes, eosinophils and natural killer cells can produce TNF-α (Tan *et al.*, 1993). It is one of the prime mediators of an inflammatory response producing a wide range of biological responses (Beutler and Cerami, 1989; Tracey, 1986). It induces fever, hypotension, leucopaenia and tissue injury. TNF-α is also responsible for the induction of neutrophils, eosinophils, monocytes/macrophages and lymphocytes and changes in vascular endothelium leading to the accumulation of white blood cells. The clinical observation that patients with severe infections and generalised wasting have high circulating

levels of TNF-α, suggest that this cytokine may be important in patients with chronic illness.

The exact role of TNF-α as a mediator in Crohn's disease or ulcerative colitis remains unclear. Circulating levels of TNF-α have been measured in several studies with conflicting results. Raised levels have been shown in both paediatric and adult populations with Crohn's disease particularly in those with active disease (Murch *et al* 1991; Maeda *et al*, 1992). However, other studies have shown normal levels in both of these populations (Hymas, 1991). The levels of soluble TNF-α receptors that have neutralising activity may explain the apparent disparity between these results. There is some evidence that there are higher levels of these receptors in the serum of patients with Crohn's disease compared to controls. The concentration of TNF-α also has been reported to be higher in the stools of patients with active IBD, with a return to normal values when the disease is in remission (Braegger, 1992). Evidence that the mucosa in IBD is the source of the TNF-α was initially found with higher numbers of cells secreting TNF-α in mucosal biopsies obtained from patients with IBD than those from normal intestine. Other studies however, have not shown consistently raised levels of TNF-α using a variety of methods such as *in situ* hybridisation (Cappello *et al.*, 1992) or PCR looking for gene transcripts (Isaacs *et al.*, 1992). It is likely that variable patient selection, tissue collection methods and diverse experimental methodology can explain some of these differences, but it would suggest that TNF-α is not consistently raised in IBD. Elevated levels also appear in other inflammatory conditions of the gastrointestinal tract, suggesting that this cytokine

may be a secondary phenomenon rather than have a primary role in the pathogenesis of IBD.

Powerful evidence that TNF-α has an important role in IBD comes with the use of monoclonal antibodies directed against TNF–α in the treatment of patients with Crohn's disease. This was initially described in two patients with severe disease in 1993 (Derkx *et al.*, 1993). Usually this treatment has been used in patients with the most severe disease i.e. steroid dependent Crohn's disease. It would appear from initial results that it is more beneficial in Crohn's disease than in ulcerative colitis (Baert *et al.*, 1999; Targan *et al.*, 1997). This is especially true for those patients with fistulating Crohn's disease (Targan *et al.*, 1997; Rutgeerts *et al.*, 1999; Ricart and Sandborn, 1999; Lofberg, 1999; Mouser and Hyams, 1999; D'Haens *et al.*, 1999). Such is the efficacy in patients with Crohn's disease that it has now become licensed for treatment in both the USA and the UK (Kornbluth, 1998). Since this initial report nearly 12000 patients have been treated with the monoclonal antibody with generally favourable results.

1.5.4. Interferon-γ

The name interferon was initially used to denote a substance capable of conferring the ability to resist against viral infection to various cells. However, the term now encompasses a large family of cytokines with both anti-viral and immune modulatory properties. This family is composed of three main types of interferon, which have been designated IFN-α, IFN-β, and IFN-γ. Each of these possesses variable degrees of anti-viral and immune modulatory activity. Interferon-γ is produced mainly by natural killer (NK) and T cells (Kawase *et al.*, 1983; Handa *et*

al., 1983). It is a weak anti-viral but a potent immune modulator. It also induces expression of the major histocompatibility complex (MHC) (Ruemmele *et al.*, 1998; Giacomini *et al.*, 1988), macrophage activation (Schultz *et al.*, 1977; Heise and Virgin, 1995), modulation of B-cell responses and enhancement of T-cell mediated cytotoxicity (Yamada *et al.*, 1990; Yeoman and Robins, 1988). The most characteristic of these functions is the induction of MHC class II molecule expression on antigen-presenting cells (Fruh and Yang, 1999; Suda *et al.*, 1995). This may be particularly important when considering the intestinal mucosa, as both classical and non-classical antigen-presenting cells are distributed throughout the mucosa.

The possible role of IFN in inflammatory bowel disease has been examined in many studies. The level in the serum of patients has been measured and also the production by peripheral blood mononuclear cells usually with inconsistent results. For example, lowered serum levels of IFN-γ have been detected in patients with Crohn's disease and ulcerative colitis (Capobianchi *et al.*, 1992). However, other studies have failed to demonstrate this. When looking at sub-populations of cells in inflamed mucosa, no spontaneous increase in IFN-γ production was seen in the supernatant of unstimulated lamina propria mononuclear cells (Lieberman *et al.*, 1988; Camoglio *et al.*, 1998). In fact, after stimulation with IL-2 or phytohaemagglutinin activation, these cells produced less IFN-γ than cells from non-inflamed mucosa (Lieberman *et al.*, 1988).

1.5.5. Interleukin-6

Elevated levels of Interleukin-6 (IL-6) in both serum and colonic mucosa have been demonstrated in IBD (Mahida *et al.*, 1991a). This is confirmed by the detection of IL-6 mRNA transcripts in actively inflamed tissues from IBD patients (Woywodt *et al.*, 1994). IL-6 positive IBD specimens were also found positive for IL-1ß and it has been suggested that the expression of IL-6 may provide an additional mediator in IBD (Stevens *et al.*, 1992). Also in common with IL-1β and other mediators IL-6 could lead to the inflammatory response characteristic of IBD, or IL-6 could play a protective role by inducing hepatic acute phase proteins. Organ cultures of involved IBD mucosa spontaneously produced increased amounts of IL-6 compared to normal mucosa (Hosokawa *et al.*, 1999). A significant relationship between serum IL-6 antigen and platelet count has been reported in IBD. It has been suggested that IL-6 may stimulate megakaryocyte activity and result in thrombocytosis in disease characterised by chronic inflammation (Heits *et al.*, 1999). Taken together these reports suggest that elevated serum IL-6 levels may be associated with the elevated platelet counts frequently seen as part of the acute phase response in IBD patients.

1.5.6.Interleukin-4

Interleukin-4 (IL-4) was originally identified in 1986 by the functional screening of a mouse T-helper cell cDNA library (Lee *et al.*, 1986; Noma *et al.*, 1986). The human equivalent was then isolated from a concanavalin-A stimulated human T-cell cDNA library based on homology with the murine sequence (Yokota *et al.*, 1986). It was initially named B-cell stimulatory factor-1 or B-cell growth factor-1on the discovery of its capacity to promote the entry of resting B-cells into the S-phase of the cell cycle (Rabin *et al.*, 1986; Paul and Ohara, 1987). The

human gene is located on chromosome 5 within 50 kilobase pairs of the gene for IL-13 (Arai *et al.*, 1989). It is secreted as a 154 amino acid precursor that is subsequently cleaved to produce a 129 amino acid secreted peptide with a molecular weight of a 15 kDa. IL-4 is primarily produced by T-cells although mast cells also produce it (Brown *et al.*, 1987). It has since been discovered that IL-4 has a wide range of activities such as the augmentation of MHC class II antigen expression on B-cells (Stuart *et al.*, 1988), stimulation of mast cells and T-cells (Mosmann *et al.*, 1986), modulation of NK cells (Conlon *et al.*, 1989) and stimulation of haemopoietic cells (Peschel *et al.*, 1987). It is known that IL-4 inhibits the release of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 from monocytes (Te Velde *et al.*, 1990), increases the production of IL-1ra (Jenkins and Arend, 1993). Probably the most biologically important effect of IL-4 is its modulation of antibody production and its role in the allergic response (Saleem *et al.*, 1998; Callard *et al.*, 1991).

Despite the variety of effects on several different immune cells and functions relevant to the modulation of immunity and the possible down-regulation of the immune response, little is known about the role of IL-4 in IBD. IL-4 has been shown to modulate the proliferation and cytotoxicity of lamina propria mononuclear cells. This effect is differentially expressed in the different forms of IBD, with IL-4 inhibiting lymphokine activitated killer cells in ulcerative colitis and in control patients but not in Crohn's disease patients (West *et al.*, 1996). Defective deactivation of intestinal macrophages by IL-4 has also been reported in IBD patients. IL-4-mediated regulation of mononuclear phagocyte effector functions is disturbed in IBD (Schreiber *et al.*, 1995a).

1.5.7.Interleukin-10

Interleukin-10 is a 35 kD homodimeric cytokine, which was originally described as Th2-cell-derived cytokine (Fiorentino *et al.*, 1989). Recent studies have shown that IL-10 is produced by a variety of cells, including Th2 cells, mast cells and cells of the macrophage lineage. This cytokine at concentrations of 10 ng/ml or less is an effective inhibitor of cytokine generation by monocytes stimulated with IFN-γ or LPS and thus it was originally known as Cytokine Synthesis Inhibitory Factor (CSIF). It acts as a suppressor through the deactivation or inhibition of monocytes and macrophages (De Vries, 1995). This would suggest the likelihood of an important role of IL-10 in the pathogenesis of chronic inflammatory diseases (de Waal Malefyt et al, 1991). This was strengthened with the discovery that mice deficient in IL-10 gene, so called "knockout" mice spontaneously develop an enterocolitis (Kuhn *et al.*, 1993). This supported the theory that IL-10 (or rather the lack of it or an effective form of it) is a key factor in the evolution of chronic gastrointestinal diseases. A possible explanation of the continuance of inflammation in IBD is the abolition of tolerance of bacterial inflammation. This inhibition of tolerance of bacterial flora is seen in experimentally induced colitis in IL-10 deficient mice, which is restored when IL-10 is infused (Duchmann *et al.*, 1996).

Using PCR, mRNA for IL-10 has been detected in human intestinal mucosa. Actively inflamed mucosa apparently contains very high levels of IL-10 mRNA (Nielsen *et al.*, 1996). Again whether this is a primary or a secondary phenomenon is as yet unclear. Patients with both UC and Crohn's disease have high circulating levels of IL-10 (Kucharzik *et al.*, 1995). This has led to the suggestion that IL-10 may act as an anti-inflammatory agent in IBD and thus

studies have been performed using IL-10 in the treatment of IBD. These studies have generally shown that in ulcerative colitis remission can be induced with IL-10 although little effect is seen with CD (Schreiber *et al.*, 1995b).

1.5.8. Interleukin-13

IL-13 is a 132 amino acid protein secreted predominantly by activated CD4+ T cells. The human gene is located on chromosome 5 (McKenzie *et al.*, 1993). It is known to be a potent suppressor of IL-1α, IL-1β, IL-6, IL-8, MIP-1α and TNF-α produced by activated monocytes and macrophages (Minty *et al.*, 1993), (McKenzie *et al.*, 1993), (Zurawski and De Vries, 1994), (de Waal *et al.*, 1993) and endothelial cells (Marfaing-Koka *et al.*, 1995). In addition IL-13 induces the production of IL-1 receptor antagonist (Muzio *et al.*, 1994) and modulates the expression of cell surface proteins such as class II MHC antigens (de Waal *et al.*, 1993). The results about the IL-13 production in IBD patients are contradictory so far (Radford-Smith and Jewell, 1996). The inhibitory effect of IL-13 on TNF-α and IL-6 production in differentiated macrophages was diminished in IBD patients and the anti-inflammatory activity of IL-13 was found partially reduced in patients with active IBD (Kucharzik *et al.*, 1996), while IL-13 has been found to inhibit nitric oxide production in activated colonic epithelial cells (Kolios *et al.*, 1998).

Although IL-4 and Il-13 are known to share the same cellular receptor (Gauchat *et al.*, 1997) and many biological functions, there are known to be some important differences between the two cytokines. The most noticeable is that IL-13 does not exhibit the ability of IL-4 to induce T cell proliferation (de Waal *et al.*, 1995;Spits *et al.*, 1987). Similarly, regulation of production of the two cytokines is not identical. Following stimulation of peripheral blood T cells, production of IL-13

mRNA is rapidly induced and long lasting whereas IL-4 production is of much slower onset and is more transient (de Waal *et al.*, 1995). IL-13 production by T-cells is inhibited by CD3 ligation and is enhanced by cyclosporin whereas the converse is true for IL-4 (Van der Pouw Kraan TC *et al.*, 1996).

1.5.9.Summary

The aetiology of IBD appears to be immunologically mediated. Cytokines with their inflammatory and regulatory properties appear to play a role in pathogenesis of UC and CD. Some cytokines have protective effects that are important in the resistance to infectious agents and possibly in the healing phase of inflammation and an imbalance of the inflammatory and protective properties of the cytokine network may determine why the inflammation becomes chronic and the tendency of these diseases to flare up again.

The evidence accumulated so far would suggest that Crohn's disease has a T helper type 1 response (Th1). Th1 cells secrete interferon-γ, lymphotoxin and interluekin-2, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-10. There is a single report that IL-4 transcripts are elevated in the early ileal lesions of recurrent Crohn's disease (Desreumaux *et al.*, 1997) but this may be a transient response in special circumstances, and as already stated IL-6 does seem important in Crohn's disease. Initial evidence suggests that ulcerative colitis is more likely to be a Th2 response as there are features suggestive of an antibody mediated autoimmunity, although the evidence for this is weak. There is the association with primary sclerosing cholangitis, another antibody mediated disease, and some patients also have autoantibodies to the perinuclear component of neutrophils (p-ANCA) (Saxon *et al.*, 1990). The inflamed bowel is filled with IgG plasma

cells and IgG co localising with complement C3b can be seen on the surface of epithelial cells (Halstensen *et al.*, 1993). Against the idea of this being a Th2 response there are groups that have shown that IL-4 is actually decreased in UC (Karttunnen *et al.*, 1994; West *et al.*, 1996). CD4 cells from patients with UC produce large quantities of IFN-γ as well as IL-5.

It also seems that environmental factors, for example dietary or microbial, trigger an inappropriate mucosal inflammatory response in individuals having either a genetically abnormal immune system, gut permeability, mucus and/or colonocyte metabolism. Although some clues about the pathogenesis of IBD are slowly becoming apparent, the mechanisms underlying why the mucosal inflammation becomes chronic and relapses of quiescent disease in patients with IBD are unknown. For example, is the chronicity of the inflammation due to a persistence of a stimulus of inflammation? Is it due to a down-regulation of the anti-inflammatory side of the immune response? (Podolsky, 1991) If we are to improve our understanding of the pathogenesis of IBD, and thus its treatment then these questions need to be answered.

1.6. Chemokines

1.6.1. Structure and Function of Chemokines

The discovery and characterisation of interleukin-8 (IL-8) revealed the existence of a large sub-group of inducible, pro-inflammatory cytokines (Baggiolini *et al.*, 1989). Chemokines are generally small heparin-binding polypeptides, which are involved in attracting effector cells to the site of inflammation and are also responsible for activating specific leukocytes populations (Rollins, 1997; Lindley *et al.*, 1993). A wide variety of cell types e.g. immune cells, fibroblasts,

endothelial cells and mesangial cells, are known to express and secrete these cytokines when stimulated e.g. by IL-1, TNF, IFN-γ, LPS (Baggiolini *et al.*, 1994). Chemokines are structurally related by possessing four conserved cysteine residues that form two disulphide bonds and establish the tertiary structure of the protein. They are then divided into sub-families based on the arrangement of the conserved cysteine residues in the molecule (Oppenheim *et al.*, 1991). Four groups of chemokines have now been defined.

The C-C sub-family in which the first two cysteine residues are adjacent, include monocyte chemotactic protein-1 (MCP-1) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES)) and preferentially act on mononuclear cells. MCP-1 is a potent chemoattractant for monocytes and macrophages (Leonard and Yoshimura, 1990) as well as for $CD4^+$ and $CD8^+$ T lymphocytes (Loetscher *et al.*, 1994; Taub *et al.*, 1995). In addition to monocytes, RANTES is a selective chemotactic agent for $CD4^+$ T cells (memory T cells) (Schall *et al.*, 1990) and eosinophils (Kameyoshi *et al.*, 1992).

The C-X-C sub-family, with IL-8 the prominent member, has an aminoacid residue between the first two cysteine residues and its primary function is that it attracts and activates neutrophils (Baggiolini *et al.*, 1989).

The discovery of lymphotactin (Kelner *et al.*, 1994) has shown the existence of a third group, named C subfamily, which lacks two (the first and third) of the four conserved cysteine residues. Lymphotactin is a chemotactic cytokine for lymphocytes, but not neutrophils and monocytes and it is produced abundantly by $CD8^+$ T cells (Kennedy *et al.*, 1995).

Finally, the recently defined CX_3C chemokine e.g. fractalkine, is an integral membrane protein with a chemokine domain in its N-terminus thus denoting a new chemokine group. This subfamily differs from the other chemokines by the presence of three amino acids intervening between the first two cysteine residues (Bazan *et al.*, 1997).

The C-X-C subfamily is further divided into those with or without an ELR motif, preceding the first cysteine residue. This motif consists of three aminoacids Glu-Leu-Arg near the amino terminus and this plays a crucial role in receptor-ligand interactions on neutrophils. Chemokines with the ELR motif (IL-8, Gro-α, Gro-β, Gro-γ, ENA-78 and NAP) act primarily on neutrophils as chemoattractants and activators. In addition, the ELR containing CXC chemokines are known to induce angiogenesis. C-X-C chemokines which lack the ELR domain (MIG, IP10 and PF-4) have no neutrophil chemotactic activity and are angiostatic (Strieter *et al.*, 1995).

Accumulation of leukocytes in the extravascular space requires a series of co-ordinated signals, including the expression and activation of cellular adhesion molecules as well as the generation of chemotactic gradients by the cells in the extravascular compartment (Huber *et al.*, 1991). Chemokines are key to several levels of this multi-step process, which requires four sequential steps: rolling, triggering, adhesion and migration (Adams and Shaw, 1994). Initially, leukocytes become attached to the endothelial layer and then roll in a slower flow along the vessel wall, thus enabling them to sample the environment for further stimuli. Selectins, which are expressed in both endothelium and leukocytes, are known to

mediate the rolling of leukocytes. However, without a further triggering factor leukocytes will detach themselves from the vessel wall (Springer, 1994).

The next step that promotes strong adhesion is due to integrin adhesiveness and again involves chemokines (Vaddi and Newton, 1994). The stimulated leukocytes spread and become firmly attached to the endothelial layer through the interaction of integrins on the leukocytes with adhesion molecules on the endothelial cells (Adams and Shaw, 1994). Interleukin-8 is an important example of a neutrophil triggering factor (Rot, 1992), while both RANTES and MIP-1β activate the T cell integrins, VLA-4 (Very Late Antigen 4) and LFA-1 (Leucocyte Function Antigen-1) (Gilat *et al.*, 1994). After this strong adhesion to the endothelium, leukocytes will then migrate into the underlying tissue along a chemotactic gradient. Differential migration of subsets of leukocytes into tissue depends on the type of integrin expressed on the leukocytes and on the interaction with a specific adhesion molecule on the endothelial surface. This expression in turn depends on the type of endothelium involved and on the stimulating chemokine. This complex mechanism allows migration of the correct leukocyte to a specific site.

1.6.2.The Role Of Chemokines In IBD

IL-8 is likely to be important in IBD as this cytokine induces neutrophil chemotaxis and thus may explain the accumulation of large number of neutrophils in the intestinal inflamed mucosa in IBD (Cole *et al.*, 1996). High levels of IL-8 are found in inflamed colonic mucosa, from patients with UC and CD compared to controls and IL-8 mRNA has been found in colonic biopsies from IBD patients, using PCR amplification and in situ hybridisation (Daig *et al.*, 1996; Isaacs *et al.*, 1992). *In situ* hybridisation revealed strong IL-8 signals in the affected mucosa in active IBD,

but not in the mucosa of uninvolved bowel segments or in normal mucosa from controls. Results from patients with acute appendicitis found IL-8-expressing cells in the mucosa suggesting that IL-8 is not specific in the pathogenesis of IBD, but it is involved in many different types of intestinal inflammation (Mazzucchelli *et al.*, 1994). Because human colonic epithelial cells have been shown to express IL-8, it has been suggested that these colonic epithelial cells may contribute to neutrophil extravasation and tissue infiltration in intestinal inflammation (Brynskov *et al.*, 1992).

Colonic epithelial cell lines have been shown to be a significant source of IL-8 after stimulation by pro-inflammatory cytokines e.g. IL-1α and TNF-α or lipopolysaccharide (Eckmann *et al.*, 1994; Schuerer-Maly *et al.*, 1994; Gross *et al.*, 1995).

RANTES, another monocyte attracting C-C chemokine, was found to be increased in the intraepithelial lymphocytes in the lamina propria of patients with IBD compared with controls (Mazzucchelli *et al.*, 1996). Using in situ hybridisation, it was demonstrated that the monocyte attracting chemokines, RANTES, MIP-1α and MIP-1β were expressed by macrophages, T lymphocytes, and endothelial cells in actively inflamed tissue but rarely expressed in non-inflamed sections from IBD. The frequency of chemokine-expressing cells was significantly greater in severely inflamed than in moderately or mildly inflamed tissue. For example, RANTES was expressed by T lymphocytes in lamina propria from normal colon, although in very small amounts (Grimm and Doe, 1996). The increased number of MCP-1 and RANTES mRNA-expressing cells in mucosa from patients with IBD suggests that these cytokines play a role in the recruitment of peripheral blood monocytes and the expression of the MCP-1 gene in vessel-associated cells may

indicate its involvement in mechanisms regulating the adhesion of blood monocytes to endothelial cells (Mazzucchelli *et al.*, 1996).

This suggests that colonic epithelial cells are important in chemokine production and may be especially important in the early recruitment of circulating immune cells in intestinal inflammation.

1.7. Other Inflammatory Mediators

The term eicosanoid is used to describe breakdown products from the membrane phospholipids into arachadonic acid. These subsequently form bioactive substances with both pro-inflammatory and anti-inflammatory activity, which include prostaglandins, thromboxanes and leukotrienes. These appear to be important in the pathophysiology of IBD (Yang, 1996) as concentrations of all the eicosanoids have been found to be significantly increased in gut lavage fluid from patients with IBD (Hommes *et al.*, 1996). Efforts to manipulate this have been made by inhibiting the production of potentially damaging eicosanoids in patients with IBD. Diets with fish oil are known to change intestinal eicosanoid synthesis, however, its effectiveness in patients with active or inactive inflammatory disease is controversial (Casellas and Guarner, 1996).

1.7.1.Prostaglandins.

Prostaglandins (PG) were the first eicosanoids to be studied in IBD, their increased production in active disease demonstrated by *in vitro* culture of colorectal biopsies and *in vivo* rectal dialyses (Rampton and Hawkey, 1984; Lauritsen *et al.*, 1989; Baumeister *et al.*, 1996). A link between eicosanoids and IBD was initially discovered when it was discovered that the mucosa of patients

with IBD contained high levels of prostaglandins whose synthesis decreased with the use of sulphasalazine (Sharon *et al.*, 1978). Luminal release of PGE_2 has been found to be significantly higher in patients with UC than in patients with CD, Clostridium difficile infection or control subjects (Lauritsen *et al.*, 1988). Furthermore, there was a significant increase in luminal PGE_2 in first-degree relatives and this suggested that increased synthesis of prostaglandins might constitute an abnormal response because of a genetic predisposition (Ahrenstedt *et al.*, 1994). The increased PGE_2 production found in the inflamed mucosa in active UC may be caused by activated macrophages and eosinophils (Raab *et al.*, 1995). Because increased prostaglandin synthesis can cause changes in both intestinal motility and by inducing mucosal secretion of water and electrolytes it could account for the diarrhoea seen in IBD (Bern *et al.*, 1989). It would also have a protective effect on the mucosa by increasing mucus secretion, altering mucosal blood flow and suppressing immune and inflammatory cell function (Rampton and Hawkey, 1984; Lauritsen *et al.*, 1989; Rampton and Collins, 1993). The importance of eicosanoids in IBD could be the explanation why NSAIDs can cause the adverse effects in IBD by inhibition of prostaglandin synthesis (Davies, 1995).

1.7.2.Leukotrienes

Another group of the eicosanoids, the leukotrienes, are involved in the inflammatory cascade in IBD. Leukotrienes are likely to contribute to mucosal inflammation particularly by recruitment and activation of neutrophils. Both *in vitro* and *in vivo* studies have suggested that increased mucosal production of leukotrienes occurs in patients with active IBD (Rampton and Hawkey, 1984;

Lauritsen *et al.*, 1989; Baumeister *et al.*, 1996; Cole *et al.*, 1996). Colonic biopsies from patients with active IBD have shown a significantly increased level of Leukotriene B_4 (LTB_4) compared with biopsies from control patients. However, in those patients receiving steroids or 5-ASA products, LTB_4 was markedly decreased. It has been postulated that drugs such as may potentially work by normalising levels of these pro-inflammatory substances (Schmidt *et al.*, 1995). Selective leukotriene inhibitors and receptor antagonists are currently under evaluation in the treatment of IBD (Roberts *et al.*, 1997). One of these, Zileuton, a 5-lipoxygenase inhibitor has however been found to be only of very minimal benefit in IBD (Hawkey *et al.*, 1997). This suggests that although leukotrienes are important in IBD, they are only one of a large number of potential mechanisms that the immune system can employ to cause inflammation.

1.7.3.Thromboxanes.

The pro-inflammatory effects of thromboxanes are both direct (recruitment and activation of neutrophils, mucosal ulceration, reduction of suppressor T-cell activity) and indirect (vasoconstriction, platelet activation) (Rampton and Collins, 1993) Thromboxanes are produced in excess in CD in inflamed mucosa, non-inflamed bowel and also by peripheral blood mononuclear cells (Rampton and Collins, 1993). It is possible that enhanced thromboxane production is important in IBD, because increased platelet aggregation and activation may be responsible for the microvascular infarction this has been proposed as an early pathogenic factor in CD (Soderholm *et al.*, 1999; Collins *et al.*, 1994). Selective thromboxane synthesis inhibitors and receptor antagonists such as picotamide and ridogrel are

available for use in man and each has been found to be of limited value in IBD (Collins *et al.*, 1994; Casellas *et al.*, 1995).

1.7.4 Platelet Activating Factor

The role of platelet activating factor (PAF) is not well understood and the evidence is somewhat contradictory. In two studies increased synthesis of PAF in the colorectal mucosa correlates well the degree of inflammation of patients with UC (Guimbaud *et al.*, 1995; Wardle *et al.*, 1996). However, other studies have found low concentrations of PAF in the rectal mucosa of patients with active UC compared with patients in remission or controls. This suggests that PAF is not an important inflammatory mediator in UC (Almer *et al.*, 1996). PAF stimulates mucosal chloride secretion in animal models and this suggests that it could contribute to the diarrhoea of UC (Bern *et al.*, 1989). The PAF receptor antagonist lexipafant shows some efficacy in treating inflammation in an animal model of acute colitis. This is demonstrated by a dose-dependent fall in macroscopic mucosal damage, neutrophil infiltration and reduced generation of inflammatory mediators (Meenan *et al.*, 1996). However, there have been no published reports of trials of PAF antagonists in human IBD, and the importance of this mediator in the pathogenesis remains unclear.

1.7.5 Biologically Active Amines

Increased histamine secretion could contribute to vasodilatation, increased vascular permeability and increased fluid and electrolyte secretion in intestinal inflammation. Many years ago work describing increased rectal mucosal synthesis of histamine in patients with UC was published (Rampton *et al.*, 1980). More

recent work has shown that histamine secretion has been found to be significantly increased in the inflamed mucosa of patients with CD and UC but not in unaffected tissue or in healthy controls. This would suggest that mast cell mediators like histamine could play a role in the pathogenesis of these diseases (Raithel *et al.*, 1995), however no efficacy of antihistamines or any mast cell stabilising agents has ever been shown.

1.7.6 Reactive Oxygen Metabolites

In IBD there is an abundant infiltration of neutrophils into the mucosa. These cells in IBD have been recognised as the main source of potentially toxic molecules known as reactive oxygen metabolites (ROMs). Increased production of ROMs is found in inflamed tissue, isolated intestinal macrophages and peripheral blood monocytes from patients with IBD (Gross *et al.*, 1994). Levels of endogenous antioxidants in patients with IBD, such as superoxide dismutase, were also found to be reduced (Salim, 1992). Indirect evidence of ROMs-mediated tissue damage comes from detection of lipid peroxidation in mucosal biopsies in active UC (Ahnfelt-Ronne *et al.*, 1990). ROMs have a wide range of pro-inflammatory actions that are likely to contribute to the pathological appearances seen in IBD. There are reports of the beneficial effects of antioxidant agents such as superoxide dismutase (Emerit *et al.*, 1989), allopurinol and dimethylsulphoxide (Salim, 1992) in IBD. Drugs such as aminosalicylates have had some of their anti-inflammatory actions ascribed to their ability to scavenge superoxide radicals directly or to inhibit the production of ROS in stimulated neutrophils (Allgayer *et al.*, 1994).

1.8.Nitric Oxide

1.8.1.Synthesis And Function Of Nitric Oxide

Nitric oxide (NO) is synthesised from the amino acid L-arginine by a family of enzymes referred to as the nitric oxide synthases (NOS). These enzymes assist in the oxidation of the amino acid L-arginine that in turn produces NO and L-citrulline. NO has a very short half-life and is broken down into other nitrogen oxides such as nitrite (NO_2^-) and nitrate (NO_3^-), and in the presence of superoxide anion to the toxic metabolite peroxynitrite ($ONOO^-$) (Moncada and Higgs, 1993). Three isoforms of the enzyme NOS have so far been identified. Two of them are continuously present and are termed constitutive, endothelial NOS (eNOS) and neuronal (nNOS), while a third isoform is only expressed in areas of inflammation. Tissues express this isoform after induction by pro-inflammatory cytokines, microbes, and bacterial products. This isoform is called inducible nitric oxide synthase (iNOS) (Knowles and Moncada, 1994). All isoforms of NOS require various cofactors e.g. nicotinamide adenine dinucleotide (NADH) and tetrahydrobiopterine (BH_4), but vary in their dependence on Ca^{2+} and calmodulin. Neuronal NOS, and endothelial NOS, which were cloned from rat cerebellum (Bredt *et al.*, 1991), bovine and human vascular endothelial cells (Lamas *et al.*, 1992; Janssens *et al.*, 1992), respectively, are all constitutive enzymes and are calcium and calmodulin dependent. Neuronal NOS and endothelial NOS share ~60% homology at the amino acid level. iNOS was originally cloned from murine macrophages and it is ~50% homologous to endothelial NOS (Lowenstein *et al.*, 1992). Constitutive NOS produce small amounts of NO, (nM quantities), and are thought to be involved in homeostatic processes such as the tone of blood vessel vasculature (Moncada *et al.*, 1991). The inducible NOS is responsible for NO

production in large quantities (μM quantities) and is highly regulated by cytokines (Moncada and Higgs, 1993; Morris, Jr. and Billiar, 1994). NO production synthesised by iNOS is delayed by several hours following stimulation, but once induced, is active for many hours. Human iNOS has been cloned and its expression and activity has been induced in a wide variety of cell types e.g. monocytes/macrophages (Reiling *et al.*, 1994), colonocytes (Sherman *et al.*, 1993), mesangial cells (Nicolson *et al.*, 1993), lung epithelial cells (Robbins *et al.*, 1994;Asano *et al.*, 1994), hepatocytes (Geller *et al.*, 1993), astrocytes (Lee *et al.*, 1993), chondrocytes (Palmer *et al.*, 1993) and smooth muscle cells (Junquero *et al.*, 1992) when stimulated with a mixture of pro-inflammatory cytokines. The activity of iNOS is sensitive to inhibitors of DNA transcription and protein synthesis, e.g. actinomycin D and cycloheximide. Other substances such as glucocorticoids (Radomski *et al.*, 1990; Pfeilschifter, 1991) and cytokines such as TGFβ, IL-4, IL-8 and IL-10 (Ding *et al.*, 1990; Cunha *et al.*, 1992; McCall *et al.*, 1992; Oswald *et al.*, 1992) have been found to inhibit iNOS activity in some cellular systems without affecting constituative NOS.

Nitric oxide (NO) has both physiological and pathophysiological actions and is thought to have both anti-inflammatory and pro-inflammatory properties depending on the type and phase of the inflammatory reaction (Moncada *et al.*, 1991; Whittle, 1995). For example, in the central nervous system NO acts as neurotransmitter and participates in functions such as long-term memory formation. A steady production of NO maintains the cerebral vasculature in a dilated state, thus regulating cerebral vascular tone.

In the inflammatory reaction induction of NO synthesis is a key part of the host response. Induced NO can have a variety of effects depending on the amount, duration, and anatomic site of synthesis. This can be advantageous or harmful depending on various factors. NO is known to be responsible for mediation of macrophage cytotoxicity (Jorens *et al.*, 1995) and inhibition of platelet aggregation. This means it may contribute to certain disease states (Kuo and Schroeder, 1995; Moncada *et al.*, 1991).

The effects of NO are widespread. Production of NO in large amounts can inhibit key enzymes by nitrosylation of reactive groups, such as iron-sulphur centres (Fe-S) and thiol groups (-SH), which are essential for enzyme catalytic function (Vedia *et al.*, 1992). NO can also inhibit enzymes in the mitochondrial electron transport chain and citric acid cycle, which may account for the cytotoxic and cytostatic effects of macrophage-derived NO on tumour cells and microorganisms. The cytotoxic activity of NO is thought to be due to the generation of more toxic radicals through its reaction with superoxide, forming peroxynitrite. This degrades to form hydroxyl radicals e.g. peroxynitrite (Beckman *et al.*, 1990). This is considered a potent oxidant that reacts with proteins, lipids, and DNA, and it is a potent initiator of DNA strand breakage, which is an initiates the activation of the nuclear enzyme poly ADP ribosyl synthetase (PARS). Activation of this enzyme in turn leads to cell necrosis or apoptosis, and probably contributes to cellular injury in inflammation. (Szabo *et al.*, 1996).

1.8.2. The Role Of Nitric Oxide (NO) In Inflammatory Bowel Disease

Nitric oxide (NO) is produced at many sites in the gastrointestinal tract and it is believed to act in pathological events (Whittle, 1994; Jorens *et al.*, 1995). The

evidence, both in animal models and in human studies, indicates that NO is involved in gastrointestinal inflammation and has a role in the pathogenesis of IBD (Boughton-Smith, 1994). Recent studies have shown a marked increase in NO synthase activity in the inflamed mucosa from patients with active UC compared to non-inflamed tissue. Increased concentrations of citrulline, formed along with NO, were found in rectal biopsy specimens from patients with active UC than in those patients with quiescent disease or normal controls. In biopsy specimens incubated with the NOS inhibitor, L-NMMA, citrulline concentrations were significantly lowered (Middleton *et al.*, 1993). Studies of the activity of both cNOS and iNOS in colonic mucosa and muscle tissue from patients with active UC and CD compared to control non-inflamed tissue revealed a substantial increase of Ca^{2+}-independent NO synthase activity in UC, characteristic of iNOS activity. The iNOS activity in colonic mucosa of UC patients was substantially higher than in control mucosa. In CD patients, mucosal NOS activity did not differ from control values. There was no difference in NOS activity in colonic muscle between patients with UC and controls (Boughton-Smith *et al.*, 1993). Measuring serum nitrate levels as an indirect measurement of NO production, it was found that NO production was increased in both active UC and CD (Oudkerk *et al.*, 1995). In a different study rectal gas samples from patients with active UC was analysed using a chemiluminescence technique. NO concentrations were found to be more than 100 times higher in patients with active UC than in the controls (Lundberg *et al.*, 1994). Studies using another technique had similar results with increased luminal NO concentrations in patients with active UC but not in controls. This study revealed that a group of the UC patients were negative for NO. Further examination showed that these patients had blood visible in the

rectum and they suspected that NO, which is bound by haemoglobin, was therefore trapped in the lumen by the blood (Reynolds *et al.*, 1995). These results also suggest that the excess NO production in UC mainly occurs in very superficial mucosal layers (Lundberg *et al.*, 1994), because luminal NO measurements reflect NO production only in the superficial parts of the mucosa, because any NO production in deeper mucosal layers is probably bound by haemoglobin in blood vessels, and therefore does not reach the lumen.

1.8.3 Nitric Oxide Production By The Epithelial Cell

The exact cellular source of NO and iNOS in IBD has received little attention until recently (Alican and Kubes, 1996). Enhanced activity of the inducible form of nitric oxide synthase has been found in the mucosa of patients with UC, but not CD (Boughton-Smith *et al.*, 1993). Other studies using immunostaining and *in situ* hybridisation have demonstrated high expression of iNOS localised to the surface epithelium and crypts in the colonic mucosa from patients with UC (Godkin *et al.*, 1996; Rachmilewitz *et al.*, 1995a; McLaughlan *et al.*, 1997). Recently this enzyme was demonstrated to be exclusively synthesised in the colonic epithelial cells in patients with active IBD by two groups using immunostaining. There was no localisation of iNOS labelling to any cells in normal non-inflamed colon (Plate 1 reproduced with kind permission from Dr G Kolios) They have shown focal iNOS labelling localised to the epithelial cells in inflamed colonic mucosa in UC (Plate 2 reproduced with kind permission from Dr G Kolios), CD, and also in diverticulitis, suggesting that iNOS expression is a feature of intestinal inflammation. There was also a similar distribution of nitrosylated protein (Singer *et al.*, 1996; Kolios *et al.*, 1998).

Plate 1. A section from histologically normal human colon which has undergone immunohistochemistry to stain for inducible nitric oxide synthase. Any cells positive for iNOS are stained brown. The section shows that no cells stain brown suggesting that iNOS is not expressed in non inflamed colon. Magnification x100

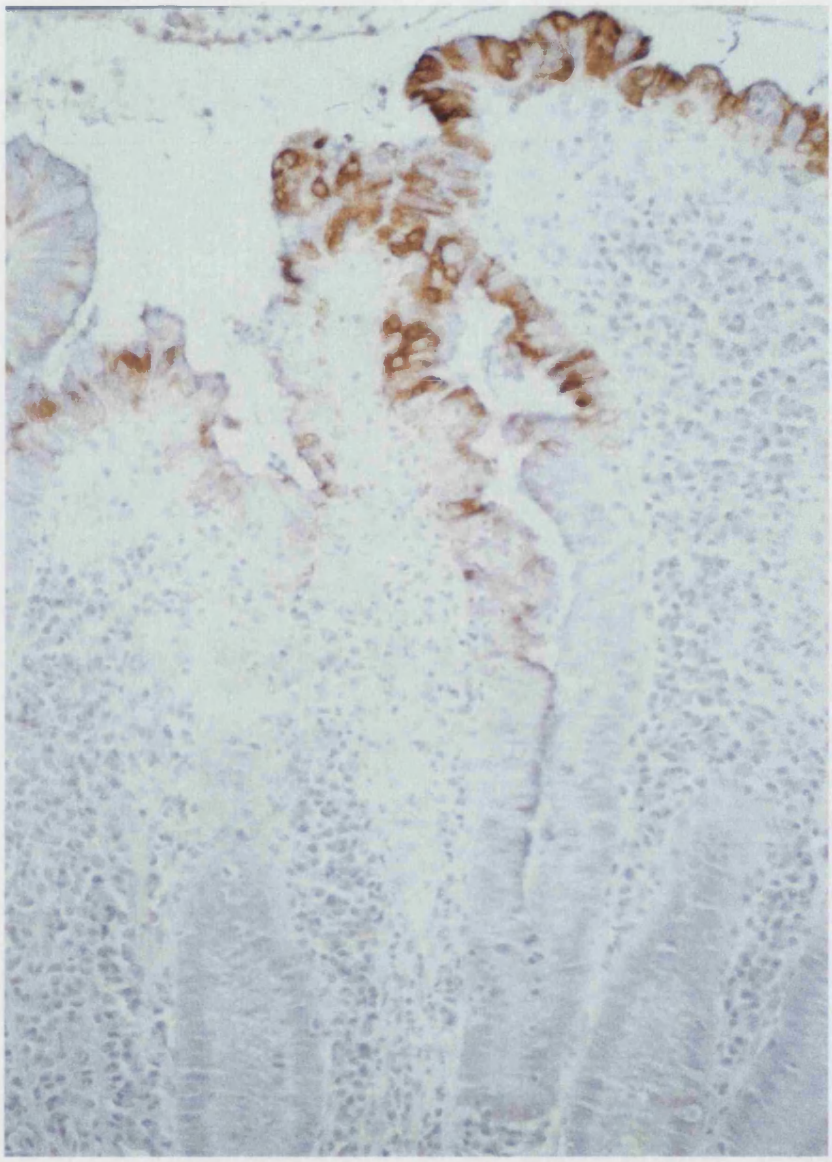


Plate 2. A section of human colon from a patient with chronic ulcerative colitis which has undergone immunohistochemistry to stain for inducible nitric oxide synthase. Any cells positive for iNOS are stained brown. The section shows that the staining is exclusively located to the colonic epithelium. Magnification x100

There does seem to be a paradox when looking at NO production. This is, that a small quantity of NO is beneficial if not essential to normal physiological functioning, but that large quantities of NO appear to be damaging. The potential mechanisms of action for the protective effects of NO include maintenance of blood flow (Cattell and Cook, 1993), inhibition of platelet and leukocyte adhesion and/or aggregation within blood vessels, modulation of mast cell reactivity, and scavenging of reactive oxygen metabolites such as superoxide (Alican and Kubes, 1996). Excess NO produced by the inducible enzyme probably exacerbates the clinicopathological features of UC by direct cytotoxicity, activation of neutrophils (Schaefer *et al.*, 1998), vasodilatation (Cohen *et al.*, 1999; Tibballs, 1993) and interaction with superoxide to form the highly toxic peroxynitrite radical (Beckman *et al.*, 1990; Kubes and Wallace, 1995). Endogenous NO produced in small quantities regulates mucosal barrier integrity and protects against the increase in mucosal permeability associated with acute inflammation. In experimental models, L-NAME protects against NO derived tissue injury and it was hoped this might be a potential new approach to treat IBD (Rachmilewitz *et al.*, 1995b).

The controlling mechanisms of iNOS production of NO by colonic epithelial cells have been studied by Dr Kolios in this group, here in Bath. Having established at the same time as Singer and his group that the epithelial cell was the principle site of iNOS production in the inflamed colon. (Plates 1 and 2) He then went on to describe how the combination of IL-1α, IFN-γ and TNF-α induces iNOS production in a colonic epithelial cell line. This was responsible for a large increase in NO production as measured by one of its metabolites, nitrite. This production could be inhibited by the T cell derived anti-inflammatory cytokines

IL-4 and IL-13, but IL-10 had no effect on this system. (Kolios *et al* 1998). There is at present little understanding of how these findings in a cell line model relate to the complex interaction of epithelial cells and other cells in the mucosa in the inflamed colon.

N-(3-(Aminomethyl)benzyl)acetamidine, also known as 1400W, is a slow, tight binding inhibitor of human inducible nitric- oxide synthase (iNOS). Inhibition is dependent on the cofactor NADPH. 1400W in these experiments was shown to be at least 5000-fold more selective for iNOS versus eNOS. Thus, the potency and selectivity of 1400W inhibition of iNOS both in vitro and in vivo were far greater than of any previously described iNOS inhibitor. (Garvey *et al.*, 1997) This has been shown to inhibit the increased production of nitric oxide as measured by nitrite by cytokine stimulated HT-29 cells (Dr S Weaver University of Bath. Personal communication of unpublished data).

1.9.Mechanism Of Action Of Therapeutic Agents

As well as knowing little if anything about the action of newer therapeutic agents e.g. IL-10, little is also known about how drugs that are used everyday in clinical practice, such as corticosteroids, act on the epithelial cell and influence the production of inflammatory mediators by the epithelial cells. Drugs such as steroids are known to have a wide variety of actions on the cellular functions that are likely to explain in part their mechanism of action. It is also apparent that the effects of steroids are widespread and act upon many components of the inflammatory process. These actions are known to include; altered lymphocyte and leukocyte trafficking, diminished adherence of leukocytes, diminished

leukocyte chemotaxis, inhibition of phagocytosis by neutrophils, inhibition of release of arachidonic acid metabolites, kinins and cytokines,

Steroids are known to form a complex with an intracellular cytosolic receptor, which then translocates to the nucleus and affects DNA transciption and protein synthesis by a variety of different actions such as inducing ribonucleases. Steroids are also known to affect gene transcription by interacting with a variety of transciption factors e.g. NFκB or AP-1(Barnes and Adcock, 1993; Brattsand and Linden, 1996; Ray *et al.*, 1995; Jonat *et al.*, 1990). Pre-treatment with glucocorticoids are known to shorten the half-life of different mRNAs such as those of IL-1β, IL-2, and IL-6.(Tobler *et al.*, 1992; Boumpas *et al.*, 1991a; Boumpas *et al.*, 1991b)

Steroids are known to have potent effects on cytokine gene transcription. In many different cell systems then steroids are known to inhibit the production of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6 IL-8 and TNF-α. (Barnes and Adcock, 1993) In colonoscopic biopsies from patients with active inflammatory bowel disease then dexamethasone has been shown to inhibit IL-1 and IL-6 (Mahida *et al.*, 1991b; Jones *et al.*, 1994)

The effect of steroids on NO production is much less clear. In many cell lines and cell types both animal and human, steroids do appear to be able to suppress iNOS e.g. the lungs (Yates *et al.*, 1995), rat mesangial cells (Pfeilschifter, 1991), Caco-2 cells (Cavicchi and Whittle, 1999), rat peritoneal neutrophils (McCall *et al.*, 1991) and RAW 264.7 cells (Walker *et al.*, 1997), although often at very large doses much higher than could ever be therapeutically obtained. Methylprednisolone has been found to decrease NO generation by cultured murine colonic mucosa although this has usually been achieved only in very high doses of

methylprednisolone. It has been suggested that NO synthase activity is reduced during the culture with methylprednisolone and this steroid effect may relate to its therapeutic effect (Rachmilewitz *et al.*, 1995a). Inhibition of NO synthesis by an L-arginine analogue significantly ameliorated the extent of tissue injury in two models of experimental colitis (Rachmilewitz *et al.*, 1995c). With the introduction of newer biological therapies such as IL-10 into clinical practice, this study sets out to examine the nature and site of action of these therapies.

AIMS

1. To investigate the regulatory mechanisms involved in nitric oxide production in human colonic epithelial cells.
2. To explore the mechanisms by which steroids and the T-cell derived cytokines IL-4, IL-10 and IL-13 act on human colonic epithelial cells to change the expression of inflammatory mediators, such as chemokines and nitric oxide.
3. To explore the effects of steroids and T cell-derived cytokines on nitric oxide and chemokine production by human colonic epithelial cells co-cultured with mononuclear cells
4. To characterise the relationship between human colonic epithelial cells and mononuclear cells to determine the factors affecting this relationship.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Cytokines

Human recombinant IL-1α This was a generous gift from Glaxo (Greenford, UK) and was diluted in sterile phosphate buffered saline (PBS) + 0.25% (w/v) bovine serum albumin (BSA, low endotoxin) (Sigma) and stored in aliquots at -70^0 C. It had a specific activity of 5x10^7 U/mg.

Human recombinant IL-4 This was purchased from Genzyme and stored in aliquots at -70^0 C. It had a specific activity > 1x10^7 U/mg

Human recombinant IL-10 This was kindly donated by Dr KW Moore (DNAX Palo Alto, California USA) and was diluted in sterile PBS + 0.1% (w/v) bovine serum albumin (BSA, low endotoxin) (Sigma) and stored in aliquots at -70^0 C. It had a specific activity 1x10^7 U/mg.

Human recombinant IL-13 This was purified from culture supernatants of stable transfected CHO cells and generously provided by Dr A. Minty (Sanofi, Recherche, Labege, France) (Minty *et al.*, 1993). It was diluted in sterile PBS + 0.25% (w/v) bovine serum albumin (BSA, low endotoxin) (Sigma) and stored in aliquots at -70^0 C.

Human recombinant IFN-γ This was purchased from Boehringer Mannheim, U.K and stored in aliquots at -70^0 C. It had a specific activity > 2.0x10^7 U/mg.

Human recombinant TNF-α This was a generous gifts from Bayer (Slough, UK) and was diluted in sterile PBS + 0.1% (w/v) bovine serum albumin (BSA, low

endotoxin) (Sigma) and stored in aliquots at -70^0 C. It had a specific activity of 6x10^7 U/mg.

Human recombinant IL-1ra This was purchased from R&D Systems Europe Ltd, U.K and stored in aliquots at -70^0 C. It had a specific activity > 2.0x10^7 U/mg.

All cell culture plastics were purchased from Nunc.

2,3-Diaminonaphthalene (DAN) was purchased from Lancaster Synthesis Ltd.

Sodium nitrite and Dimethyl-sulphoxide (DMSO) were purchased from Sigma Chemical, UK.

Antibodies for IL-8 (anti-human IL-8 mouse monoclonal antibody, alkaline phosphatase conjugated anti-human IL-8 goat polyclonal antibody and human recombinant IL-8 standard) enzyme-linked immunosorbent assay (ELISA) were a generous gift from Dr I.J.D. Lindley (Sandoz Forschungsinstitut, Vienna, Austria) (Ceska *et al.*, 1989).

2.2 Culture Conditions

2.2.1 HT-29 Colon Adenocarcinoma Cell Line

The human colon epithelial cell line HT-29 was obtained from the European Collection of Animal Cell Cultures (ECACC). The cells are adenocarcinoma grade II cells isolated from a primary tumour in a 44 year old Caucasian female (ECACC). They have characteristics of normal colonic epithelial cells such as epithelial

polarity, presence of the actin-biding protein villin and the occurrence of enterocyte differentiation (Chantret *et al.*, 1988).

2.2.2.Cell Culture

HT-29 cells were cultured in 80 cm^2 tissue culture flasks in McCoy's medium supplemented with penicillin (10u/ml), streptomycin (10μg/ml), fungizone (0.5μg/ml), and 10 % (v/v) foetal calf serum (FCS) referred to as complete medium. Cultures were then maintained at 37^0 C in an atmosphere of 5% CO_2. The complete media was changed between 48 and 72 hours. When a confluent monolayer of HT-29 cells was present the medium was removed and the cells were washed 3 times with PBS without Ca^{2+} or Mg^{2+}. Cells were then washed once with 3mls of a trypsin-EDTA mixture containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. Any excess solution was removed and the cells were incubated for approximately 5 minutes at 37^0 C and agitated gently until the cells had detached from the flask. The action of trypsin/EDTA was inhibited by adding 10ml of complete McCoy's medium and the cell suspension was centrifuged at 200g for 5 min. The cell pellet was resuspended in complete medium and cell counting and viability were checked using a Neubauer haematocytometer after mixing with Trypan Blue (Sigma). Any cells staining blue, due to the uptake of Trypan Blue were considered dead. Cell viability was always greater than 95%. Cells were counted and then seeded at 2-3 x 10^4/ml of McCoy's complete medium, into 80 cm^2 tissue culture flasks for further culture, or into 6-well plates for experimental protocols. Again media was exchanged after 48-72 hours until the HT-29 cells in the flasks and plates became confluent. This usually happened between 6-7 days after the cells were passaged.

If the cells were being stored, cells were resuspended at a concentration of 4 x 10^6 cells/ml in a medium containing 10% of dimethylsulphoxide (DMSO) (Sigma), 40% FCS, and 50% McCoy's medium. This cell suspension was then aliquoted into cryotubes (Nunc) at 1 ml / tube, gradually cooled in vapour phase of liquid nitrogen overnight and tubes were stored in liquid nitrogen. For resuscitation of cells from the liquid nitrogen, cells were rapidly defrosted at 37^0C in a water bath, washed immediately in McCoy's medium, re-suspended in complete medium and cells from 1 cryotube were seeded into 80 cm^2 tissue culture flasks in McCoy's medium, continuing as above.

When 6-well plates were confluent with HT- 29 cells, growth was arrested by changing the media to FCS-free McCoy's medium and incubating for twenty-four hours. These growth-arrested cultures were then treated with fresh FCS-free McCoy's medium and stimulated with the appropriate doses of either drugs, or cytokines, or vehicle controls for the times described in the results section. Supernatants were then collected, cellular debris removed by centrifuging the media and stored at -70^0 C until assayed for nitrite or chemokines. Cell counting and viability was routinely checked at the beginning and the end of the experiment, by microscopy and by trypan blue exclusion, using representative wells. Cell viability was always greater than 95%.

2.2.3. Colonic Biopsy Cultures

Patient Selection

Multiple colonic biopsies were taken from the sigmoid colon in patients who underwent colonoscopy at the Royal United Hospital, Bath. These biopsies were approximately 2mm in diameter and weighed approximately 5-7mg. Patients used

were divided into one of two different groups, dependent ultimately on the histological diagnosis reached by one of two experienced Consultant Histopathologists with a special interest in gastrointestinal pathology who were unaware of which patients were included in any experiments.

The patient groups were as follows:

1) Anyone with a new clinical diagnosis of ulcerative colitis who had yet to start on any treatment (n=17) (8 men and 9 women, mean age 44.9 years, range 16-81).

2) Anyone undergoing a colonoscopy for other reasons e.g. abdominal pain where the examination was found to be normal. (n=22) (9 men and 13 women, mean age 51.3 years, range 24-84) (n=10)

A further group of patients was identified as those patients with distal ulcerative colitis with endoscopically normal mucosa in the proximal colon. Biopsies from this apparently normal area were studied in separate experiments if histological examination confirmed a normal appearance to histological examination. (n=8) (5 males, 3 females, mean age 42.6 years, range 34-64)

Consent was obtained from patients in all cases and the local Research Ethics Committee granted approval for these studies.

2.2.4. Biopsy Culture

Human colonic biopsies were cultured using a method recently published by our group (Kolios *et al.*, 2000). The biopsies were immediately placed in transport medium, Hanks' balanced salts solution (HBSS) pH 7.4, supplemented with

antibiotics (penicillin 100U/ml, streptomycin 100 μg/ml, gentamicin 50 μg/ml, and fungizone 2.5 μg/ml), transferred to the laboratory, and gently washed 3 times for 15 minutes in the transport medium. Mucosal biopsy specimens were then placed in a six-well plate (Gibco), containing 2 ml of Trowell's-T8 65% (v/v) / RPMI-1640 25% (v/v) / foetal calf serum 10% (v/v) medium (supplemented with Hepes buffer 10 mM, glutamine 2 mM, penicillin 100U/ml, streptomycin 100 μg/ml, gentamicin 50 μg/ml, and fungizone 2.5 μg/ml) per well. Colonic biopsies were incubated at 37^0 C in an atmosphere of 5% CO_2 in the presence of appropriate stimuli and were cultured for 30 hours. Supernatants were then collected for nitrite measurement and colonic specimens were used for total protein estimation of each well.

2.2.5. Co Culture Of HT-29 And Mixed Mononuclear Cells

Mixed mononuclear cells (MMC) were isolated from blood from healthy human volunteers (n=4) using a standard protocol based on a method originally described by Boyum (Boyum, 1976). Briefly, for each subject a 50mls syringe was pre-heparinised with 250U of standard heparin. 50mls of blood was taken and diluted 1:1 with RPMI 1640. Approximately 35mls of the mixture was then layered onto 15mls of LymphoPrep (Nycomed, Oslo, Norway). This was then centrifuged for 30 minutes with no brake and this gave a layer of mixed mononuclear cells. These were isolated and then washed three times in RPMI 1640. They were then resuspended in McCoy's 5A media with 2% foetal calf serum supplemented with penicillin-streptomycin (10U/ml and 10μg/ml) and fungizone (0.5μg/ml). Growth arrested HT-29 cells were co-cultured with 0.5×10^6 /ml of healthy MMCs.

2.3. Fluorometric Nitrite Assay

Nitric oxide (NO) was determined by measuring the stable-end product, nitrite, in culture supernatants. Nitrite was measured using a fluorometric assay based upon the reaction of 2,3- Diaminonaphthalene (DAN) (Lancaster, Morecambe, UK) with nitrite under acidic conditions to form the fluorescent product 1-(H)-naphthotriazole. The assay was modified for use on a Photon Technology International (PTI) spectrofluorometer from the method of Misko *et al* (Misko *et al.*, 1993), which employed a 96 well plate format for a fluorescent plate reader. In contrast to the plate reader, the optimum excitation and emission wavelengths for the assay could be set on the fluorimeter, thus improving sensitivity. Fluorescent excitation and emission spectra for 1-(H)- naphthotriazole were obtained as previously described and optimum wavelengths were determined. An excitation wavelength of 365 nm and emission wavelength of 405 nm were found to be optimum, which was consistent with previous findings. A standard curve of sodium nitrite in McCoy's medium ranging from 100 nM to 2 μM was prepared. 2 ml of standard or culture supernatant was mixed in a bijou with 200 μl of freshly prepared DAN reagent (0.05 mg/ml DAN in 0.62 M HCl) and incubated at room temperature in the dark. After 10 minutes the reaction was stopped by the addition of 100 μl 2.8 N NaOH. Fluorescence intensity of 2 ml volumes of standards and samples was measured on the fluorimeter using an excitation wavelength of 365 nm and an emission wavelength of 405 nm. Phenol red present in McCoy's medium did not interfere with the assay. The lower limit of sensitivity of the assay was 10nM.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA) For IL-8

Extracellular chemokine activity of culture supernatants was measured by double ligand ELISA. All ELISA samples were measured in duplicate using a well-established method (Brown *et al.*, 1994). 96 well microtitre plates (Nunc Immuno Maxisorb) were coated with 100 µl/well of monoclonal anti-IL-8 antibody (5 µg/ml) in carbonate coating buffer. Plates were covered and incubated overnight at 4^0 C. Plates were washed 3 x with wash buffer, followed by addition in duplicate of 100 µl IL-8 standard (0.05 to 2 ng/ml) or culture supernatant appropriately diluted in wash buffer + 2 % (v/v) FCS, and incubated for 2 hours at 37^0 C. Plates were washed 3 x and 50 µl alkaline phosphatase-conjugated goat anti-IL-8 antibody (5 µg/ml) in wash buffer + 2 % FCS was added for 2 hours at 37^0 C. Following 3 washes, plates were incubated with 100 µl of 1 mg/ml *p*-nitrophenyl phosphate in warmed diethanolamine buffer at room temperature, until the top standard read ~ 1.5 OD. The reaction was terminated with 50 µl/ well of 3M NaOH, and the OD measured at 405nm. The assay was linear over a range between 0.2-2 ng/ml.

2.5. Protein Assay

To standardise for different size biopsies used in the studies the total protein of the biopsies was analysed. After 30 hours culture with appropriate stimuli culture supernatants removed for nitrite measurement and colonic biopsy specimens were used for estimation of protein content, using the Bio-Rad Protein microassay. The protein assay was based on the Bradford dye-binding procedure (Bradford, 1976). Known concentrations of bovine serum albumin (BSA) (Sigma) diluted in

phosphate buffered saline (PBS), pH 7.4 were used as a standard curve. Colonic specimens were homogenised in PBS, pH 7.4, using an ultrasonic homogeniser and solubilised tissue was diluted in various dilutions (1:10-1:200). 200 µl of sample or standard and 50 µl of Bio-Rad protein Assay (Bio-Rad) were added per well in a 96-well microtiter plate and protein was measured with a microplate reader.

2.6.Polymerase Chain Reaction

2.6.1.RNA Isolation

Total cellular RNA was isolated from HT-29 colon adenocarcinoma cells using RNAzol (Biogenesis Poole UK) according to the manufacturers instructions. Briefly the biopsies were homogenised in RNAzol (2ml per 100mg tissue) 0.1ml of chloroform per 1 ml of homogenate was then added, the samples shaken vigorously for 15 seconds and then cooled on ice for 5 minutes. The suspension was then centrifuged for 15 minutes at 12,000g at 4°C. The homogenate then forms two phases: the lower blue phenol- chloroform phase and the colourless upper aqueous phase. The aqueous phase was then transferred to a separate eppendorf and an equal volume of isopropanol was added. The samples were then stored at 4°C for 15 minutes. The samples were then centrifuged at 12,000g for 15 minutes at 4°C. RNA will then form a precipitate at the bottom of the tube. The supernatants were removed and RNA pellet washed in 1.5 ml 75 % ethanol solution. The pellet was then dried (but not completely) and then re-dissolved in RNase free water.

2.6.2. Isolation Of mRNA

Then using a commercially available kit (QuickPrep Pharmacia) Poly (A) RNA was extracted from the above samples following manufacturers instructions. The supernatant was then mixed with oligo (dT) cellulose for three minutes. Following a further centrifugation, the pellet was washed with a high salt buffer (x5) followed by a low salt buffer (x2). It was then transferred to a microspin column from which the mRNA was eluted with the elution buffer. The mRNA was then precipitated with 10µl of glycogen solution and 40µl of potassium Acetate 1 ml of 95% ethanol. The mRNA was then pelleted by centrifugation and then re-dissolved in water. mRNA was quantitated by measuring absorbance of 2 µl RNA in 1 ml 0.1 M NaOH at 260 nm. The amount of RNA present in the samples (in µg) was calculated by:

A_{260} x dilution factor (500) x 40 x volume of remaining RNA solution in ml (0.048)

ODs were also read at 280 nm and 230 nm to assess the purity of RNA. A value of less than 2 for the OD_{260} : OD_{280} ratio indicated protein contamination. A low OD_{260} : OD_{230} ratio indicated guanidine contamination.

2.6.3 cDNA Generation Using Reverse Transcription PCR

Poly (A) RNA then underwent reverse transcription to generate cDNA. Initially 5µl of the QuickPrep column elutate was diluted to a final volume of 12.5µl and denatured at 75°C for 3 minutes. The solution was then cooled at 4°C before the addition of 7.5µl of RT reaction mixture containing sufficient reagents for optimal concentration in 20µl. Random hexamers (Pd $(N)_6$) at a final concentration of 1µM, RT buffer x1, dNTPs each at 0.5mM, RNAsin at 1U/µl and reverse transcriptase (superscript) at 1µl. Reverse transcription was performed at 42 °C for 60 minutes

followed by a denaturation at 95°C for 2 minutes. The product was cooled on ice and underwent PCR immediately. To amplify fragments of human iNOS, β Actin and IL-8 35 cycles of PCR were carried out using 100ng of cDNA as a template and oligo nucleotide primers corresponding to the genes being examined.

The primer sequence for Human iNOS was TGTGTCTTGGAAAGTCATCC and AATTCCACCAGTATGCAATG. This gave a product size for iNOS of 263 Kb. These primer sequences had been designed and selected using a commercially available program (Primer3 Output) so as to minimise primer dimerisation between pairs and with the selected house keeping gene (β actin) which served as an internal standard thus allowing semi-quantitative analysis between different samples.

The primer sequence for human IL-8 was TGGGTGCAGAGGGTTGTG and CAGACTAGGGTTGCCAGATTTA. This gave a product size for IL-8 of 562 Kb.

The primer sequence for Human β actin was CTTTTCCAGCCTTCCTCC and GCAGTAATCTCCTTCTGCATC. This gave a product size for β-actin of 176 Kb.

The primers were synthesised and supplied by PE Applied Biosystems UK. (Warrington UK).

2.6.4 Separation Of PCR Products

PCR products to which 5μl of 0.25% bromophenol blue and 15%Ficoll in water, were then run on a 2% agarose gel in 0.5x TBE at 100V to which ethidium bromide had been added. Bands were detected under UV light and compared to the 100base pair ladder (Gibco). Co-amplification of both β actin and either IL-8 of iNOS were done on the same samples using a modification of a method previously described (Dr E Campbell and Dr M Watson, University of Bath. Personal communication) It had been demonstrated in earlier experiments that increasing amounts of RT product

(0.3μl, 1.5μl and 3μl) in the PCR reaction yielded differing amounts of final cDNA. This relationship could be demonstrated to be linear. Once above a certain amount of RT product then eventually a plateau in the amount of cDNA was produced. This highlighted the need to titrate each sample to allow accurate quantification. Product densities for β Actin, IL-8 and iNOS were linear in the range tested thus allowing cross sample comparison for IL-8 and iNOS relative to β-Actin. The ratio of PCR product, as measured by densitometry between IL-8 or iNOS and β actin at a constant volume of RT product (1.5μl was chosen), thus allowing standardisation of the target cDNA levels relative to those of the housekeeping gene and thus allowing cross sample comparison using the Mann Whitney U test.

2.7. Experimental Protocols

2.7.1.HT-29 Cells

HT-29 cells were seeded at 2-3 x $10^4/cm^2$ in six well plates and were maintained at 37^0 C in an atmosphere of 5% CO_2 until confluent. Prior to stimulation with pro-inflammatory cytokines cells were pre-treated for two hours with doses of one of two steroids: prednisolone or budesonide (Sigma) from 30nM to 300μM. They were then treated with concentrations of IL-1 (10ng/ml), IFN-γ (300U/ml) and TNF-α (100ng/ml) in medium without the foetal calf serum and incubated for a period of 48 hours.

2.7.2. Colonic Biopsies

Multiple colonic biopsies were taken from the sigmoid colon in patients with newly diagnosed ulcerative colitis who underwent colonoscopy prior to the patient starting on any treatment. Paired biopsies were then incubated with one of two

corticosteroids (prednisolone or budesonide) or one of three T cell derived cytokines (IL-4, IL-10 or IL-13) for 30 hours. Supernatant was removed and analysed for nitrite and IL-8. The biopsy material was analysed for protein content.

Similar biopsies were taken from the sigmoid colon in patients undergoing a colonoscopy for other reasons e.g. abdominal pain where the examination was found to be normal. Only those patients where the histology was reported as normal were included in the experiment. These biopsies were pre-treated with one of the two corticosteroids or one of three T cell derived cytokines for 2 hours. They were then stimulated with the combination of IL-1 (10ng/ml), IFN-γ (300U/ml) and TNF-α (100ng/ml) known to induce iNOS and IL-8 expression in HT-29 cells for a further 30 hours.

Further colonoscopic biopsies were taken from endoscopically normal areas in those patients with a new diagnosis of distal ulcerative colitis who had not yet been started on treatment. Only those patients where the histology was confirmed the endoscopic appearance of apparently normal mucosa were included in these experiments. These biopsies were pre-treated with either prednisolone or budesonide or one of the three T cell derived cytokines for 2 hours. They were then stimulated with the combination of IL-1 (10ng/ml), IFN-γ (300U/ml) and TNF-α (100ng/ml) known to induce iNOS and IL-8 expression in HT-29 cells for a further 30 hours.

2.8. Co-Culture Of Mixed Mononuclear Cells And HT-29 Cells

2.8.1. Examination Of A Variety Of Stimuli On The Co-Culture

To find appropriate stimuli that were able to induce NO production in a co-culture but not in a single culture of either the HT-29 cells or the MMCs, the MMCs were

stimulated both in isolation and also in a co-culture with the HT-29 cells. Nitrite was measured in the supernatant as a measure of NO production.

HT-29 cells were seeded at 2-3 x $10^4/cm^2$ in six well plates and were maintained at 37^0 C in an atmosphere of 5% CO_2 until confluent. Once confluent these were co-cultured with MMCs isolated from normal volunteers as described above. The MMCs were at a concentration of 500,000/ml. A wide variety of stimuli were used either alone or in combination. Stimuli used included IL-1 (10ng/ml), IFN-γ (300U/ml) and TNF-α (100ng/ml) and Lipopolysaccharide (LPS) (5μg/ml). The aim of this experiment was to find the simplest stimulus which would induce a large rise in NO production in the co-culture but not be able to induce NO in either of the two cell types when cultured alone.

2.8.2 Effect Of Different Pre-Treatments On Stimulated Co-Cultures

Once the simplest combination of stimuli had been ascertained that would induce nitrite production in the co-culture the effect of either one of the corticosteroids or one of the T cell derived cytokines was examined. Different doses of these pre-treatments were introduced to the co-culture. After 2 hours the MMCs were stimulated to induce NO production. Supernatant was collected after a further 30 hours and then analysed for nitrite.

2.8.3 Examination Of Conditioned Media From MMCs On HT-29 Cells

In separate experiments supernatant from stimulated MMCs (conditioned media) was removed after 6 hours and then the media was then centrifuged for 10 minutes to allow the collection of cell free media. This cell free media was then

transferred to confluent HT-29 cells in 6 well plates. After a further 24 hours the media was removed and analysed for nitrite.

2.8.4. Examination Of Site Of Action Of Different Pre-Treatments

To examine the probable site of action of the steroids or the T cell derived cytokines on the stimulated co-cultures the following experiments were performed. Either the HT-29 cells or the MMCs were pre-treated for 2 hours with one of the steroids or one of the T-cell derived cytokines (IL-4, IL-10 or IL-13). The MMCs were then stimulated as previously described and the after a further 6 hours the media was removed and centrifuged for 10 minutes to obtain a cell free media. This was then transferred to the confluent HT-29 cells. After a further 24 hours the media was removed and underwent analysis for nitrite.

A further set of experiments was performed using interluekin-1 receptor antagonist (IL-1ra). This was performed by pre-treating either the MMCs for 2 hours prior to stimulation or by addition of the IL-1ra to the conditioned media immediately prior to transfer to the HT-29 cells. The media was then removed after 24 hours and underwent analysis for nitrite.

2.9 Statistical Analysis

Duplicate determinations were performed in each experiment. The (n) number of each experiment is given in the respective figure legend. Data were analysed either by two-way analysis of variance (ANOVA) to determine if any statistical significance existed within the data groups or when using paired samples a Wilcoxon signed rank test was performed. ANOVA was followed by Dunnett's test for the comparison of multiple groups to controls. This latter test identified which

treatments within the group were significantly different from the control. Data were expressed as means ± SEM of (n) experiments. A probability value of $p < 0.05$ was taken as the criterion for a significant difference. Statistical anaylsis was done using SPSS for windows version 10.0.5

2.10 Buffers And Solutions

2.10.1.Solutions And Reagents For Cell And Tissue Culture

Tissue culture reagents were used for cell and tissue cultures and all solution were prepared using sterile, pyrogen-free distilled water (Steripak Ltd, Cheshire,UK).

Phosphate Buffered Saline (PBS), pH 7.4

140 mM NaCl

2.7 mM KCl

1.5 mM KH_2HPO_4

8.1 mM Na_2HPO_4

Hanks' Balanced Salts Solutions (HBSS), pH 7.4

100 ml 10X Hanks' balanced salts (Gibco)

6 ml 7.5 sodium bicarbonate (Gibco)

20 ml 1M HEPES buffer solution (Gibco)

200 µl 40% (w/v) NaOH

Make up to 1L with distilled water. No need to pH.

McCoy's medium 5A (Gibco)

Supplemented with penicillin (10u/ml, Gibco), streptomycin (10 µg/ml) (Gibco), and fungizone (0.5 µg/ml) (Gibco) before use.

Foetal Calf Serum (FCS)

FCS (Gibco) was heat-inactivated at 56^0 C for 30 minutes and stored at 20^0 C.

Trowell's T8 / RPMI-1640 medium

65% Trowell's T8 (Gibco)

25% RPMI-1640 (Gibco)

10% Foetal Calf Serum (FCS) (Gibco)

10 mM HEPES buffer solution (Gibco)

2 mM Glutamine (Gibco)

Supplemented with penicillin (10U/ml) (Gibco), streptomycin (10 μg/ml) (Gibco), and fungizone (0.5 μg/ml) (Gibco) before use.

2.10.2. Solutions and buffers for Nitrite assay

HCl solution

0.62M HCl in Milli-Q water.

NaOH solution

2.8N NaOH in Milli-Q water.

2.10.3. Solutions and buffers for ELISAs

Wash buffer, pH 7.3

140 mM NaCl

2.7mM KCl

1.5mM KH_2HPO_4

8.1mM Na_2HPO_4

0.05% (v/v) Tween-20

Coating buffer, pH 9.6

15mM Na_2CO_3

35mM $NaHCO_3$

3mM NaN_3

Adjust pH to 9.6 with 1N HCl. Buffer can be kept for 2 weeks.

Diethanolamine substrate buffer, pH 9.8

10% (v/v) diethanolamine (BDH)

1mM $MgCl_2$

Store in the dark, at 4^0 C.

DEPC-treated water or buffer

Milli-Q water or buffer were treated with 1 ml of diethyl pyrocarbonate (DEPC, Sigma) per litre of water, and incubated overnight at 37^0 C. Autoclave (for 20 minutes at 121^0 C).

0.5x TBE Stock (For 1 L)

Tris base 54g

Boric acid 27.5g

0.5M EDTA (pH 8) 20ml

Chapter 3

RESULTS

Effects Of Prednisolone and Budesonide on Inducible Nitric Oxide Synthase And IL-8 Expression in Colonic Epithelium

3.1 Effect Of Corticosteroids On Inducible Nitric Oxide Synthase (iNOS) And IL-8 Activity In Colonic Epithelial Cells

Growth arrested monolayers of HT-29 cells incubated for 48 hours in serum free media produce a small constitutive amount of nitrite. In these HT-29 cells, nitrite production increased from a basal level of 422 ± 38 (control ± SEM) to 1068 ± 158 pmoles/10^6 cells at 48 hours when stimulated with the three cytokines: IL-1α (10ng/ml), IFN-γ (300U/ml) and TNF-α (100ng/ml). Pre-treatment with increasing concentrations of budesonide from 10nM to 300μM for two hours prior to 3 cytokine stimulation only decreased nitrite production at very high concentrations of budesonide e.g. 30μM to 740 ± 82, 100 μM to 694 ± 88 and 300μM to 546 ± 24 pmoles/10^6 cells. Pre-treatment with increasing concentrations of prednisolone from 10nM to 300μM for two hours at increasing concentrations of up to 300μM had no significant effect on nitrite production. Treatment of the HT-29 cells with either steroid alone did not significantly change the nitrite concentration in the supernatant. (Figure 1)

Growth arrested monolayers of HT-29 cells which had been incubated for 48 hours in serum free media produce a small constitutive amount of IL-8 production of 36.7 ± 0.1 ng/ml (control ± SEM). With three cytokine stimulation this rose to 472 ± 17.2 ng/ml. Pre-treatment with increasing concentrations of budesonide from 10nM to 300μM for two hours prior to 3 cytokine stimulation only

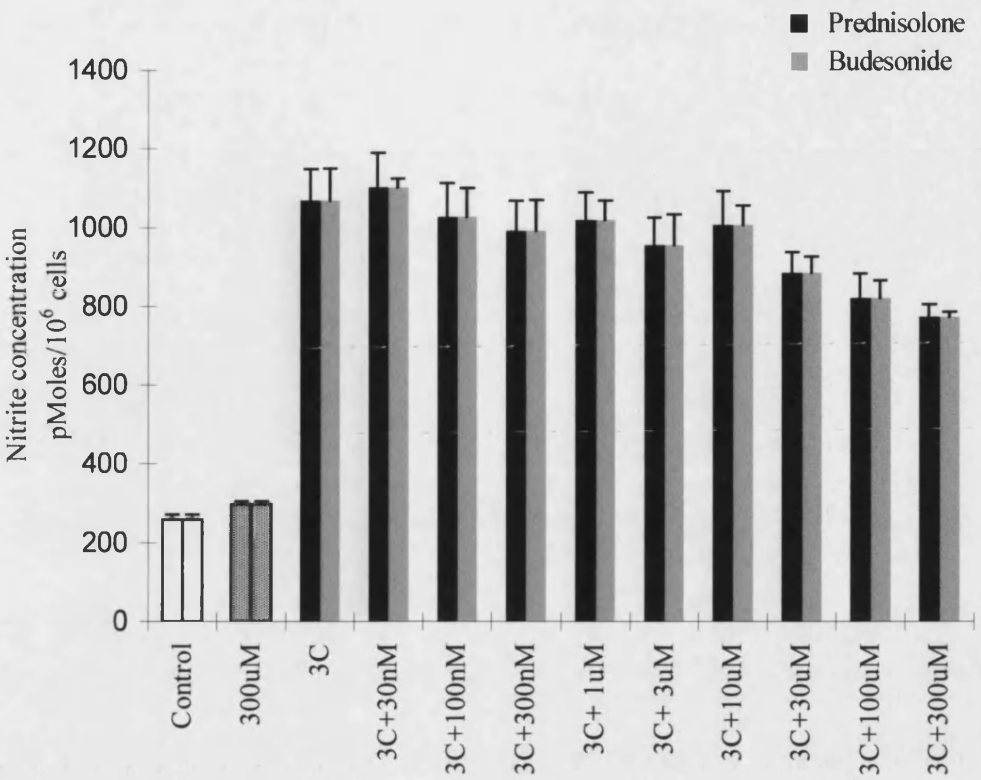


Figure 1. Nitrite production by HT-29 colonic epithelial cells following stimulation for 48 hours incubation at 37° C with vehicle and the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ ≠(300U/ml) added in combination (3C). Control is the amount of nitrite produced by HT-29 cells in the absence of added cytokines. 300μM is the effect of 300μM of either of the two steroids added alone. A series of increasing doses (30nM-300μM) of the two steroids were used to pre-treat the HT-29 cells 2 hours prior to stimulation with the 3 cytokines. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. Each bar is the mean ± SEM of four experiments. No significant effect was found at any doses of the two steroids used.

decreased IL-8 production again at very high concentrations of budesonide e.g. 100μM to 264 ± 10 and 300μM to 251.2 ± 40 ng/ml. Pre-treatment with increasing concentrations of prednisolone from 10nM to 300μM for two hours prior to 3 cytokine stimulation had no significant effect on IL-8 production. Treatment with either steroid alone at a concentration of 300μM for 48 hours did not significantly change the IL-8 production by the HT-29 cells. (Figure 2)

3.2 Effect Of Corticosteroids On iNOS And IL-8 Activity By Colonic Biopsies

3.2.1. iNOS Activity In Colonic Biopsies From Inflamed Mucosa.

To examine why the two steroids have no effect on the colonic epithelial cell line yet obviously work in clinical practice the effect of the two steroids on NO and IL-8 production by paired colonic biopsies from patients with colitis. These were patients undergoing colonoscopy in which a new diagnosis of colitis was made. Prior to the biopsies being taken, patients had not been on any regular medication at all. Only patients who had inflammation consistent with colitis reported were included in the experiments.

Mucosal biopsy specimens of each patient were placed in four wells of a six well plate containing culture media with the appropriate stimuli per well. Two of the three wells were treated with 30nM of either prednisolone or budesonide. After 40 hours total protein was estimated per well and the individual nitrite content of each well was measured and expressed as pmoles/100μg Protein. (Table 1)

At 40 hours baseline mean nitrite production per 100ug of protein in biopsies from inflamed areas in colitic patients (n=9)(M:F 6:3) was 1090 ± 351 pmoles/100μg protein (mean ± SEM). With incubation of the biopsies with prednisolone (30nM) this fell to 384 ± 176 pmoles/100μg protein ($p<0.05$). If the

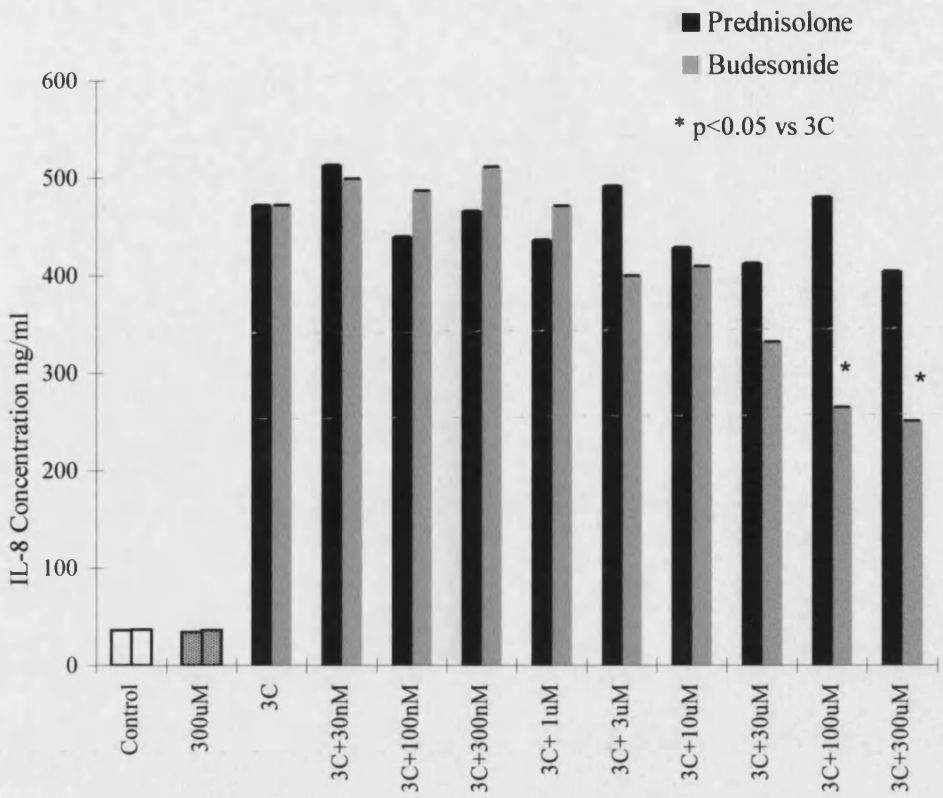


Figure 2. IL-8 production by HT-29 colonic epithelial cells following stimulation for 48 hours incubation at 37° C with vehicle and the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Control is the IL-8 concentration produced by HT-29 cells in the absence of added cytokines. 300μM is the effect of 300μM of either of the two steroids added alone. A series of increasing doses (30nM-300μM) of the two steroids were used to pre-treat the HT-29 cells 2 hours prior to stimulation with the 3 cytokines. IL-8 levels were determined in supernatants, using an ELISA specific to IL-8. Each bar is the mean ± SEM of four experiments. The only significant effect was found at doses of budesonide of 100 and 300μM ($p<0.05$).

biopsies were incubated with budesonide (30nM) production of nitrite fell to 313 ± 104 pmoles/100μg protein. ($p<0.05$) (Figure 3)

Patient (No)	Basal	Prednisolone	Budesonide
1	834	252	388
2	465	39	29
3	583	19	57
4	594	456	328
5	491	71	115
6	3655	51	15
7	1767	1116	905
8	1012	1425	695
9	403	15	283

Table 1. Nitrite production by cultures of human colonic mucosa from inflamed mucosa. Effect of incubation with 30nM of either prednisolone or budesonide. All values expressed as pmoles/100μg protein

Paired biopsies from inflamed areas in the colon in patients with colitis were incubated with either prednisolone or budesonide for 40 hours. At the end of this period the mRNA from the biopsies was extracted and then using primers for both β actin and iNOS underwent PCR. This clearly showed that RNA for β actin and iNOS was present in the biopsies (Figures 4 and 5). To estimate the effect of the two steroids on the biopsies, the RNA underwent PCR with the two different primers at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the two steroids. Levels for iNOS were compared to those for β-actin. This showed a significant fall in levels of iNOS when treated with either Prednisolone or Budesonide compared to levels in the untreated biopsies. Levels fell to 54.4% ±9.3% ($p<0.05$) when treated with prednisolone and 59.2% ±10.3% ($p<0.05$)(Figure 6). Representative blots from a patient are shown (figure 7).

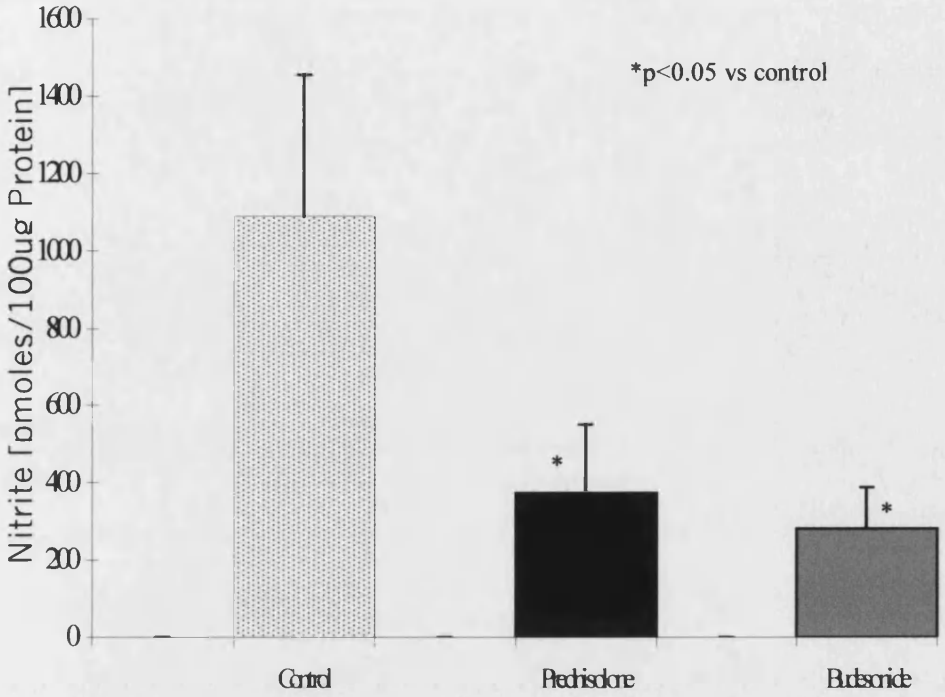


Figure 3. Nitrite production by colonic biopsies taken from inflamed areas in patients (n=9) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of nitrite produced by the biopsies in 40 hours. Paired biopsies were also incubated with either prednisolone or budesonide at a concentration of 30nM. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. Nitrite levels are expressed as pmoles in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of nine experiments. A significant effect was found at using this dose of the two steroids compared to control samples ($p<0.05$). There was no significant difference between the two steroids at the dose studied.

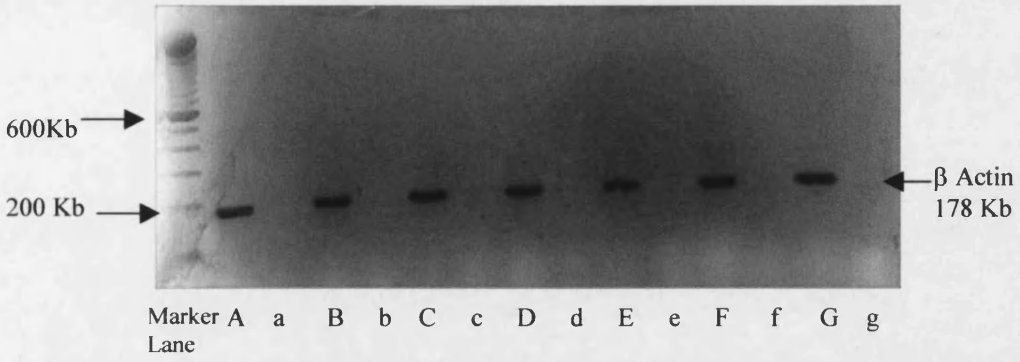


Figure 4. β-actin expression in colonic biopsies from 7 patients with ulcerative colitis (A-G). Lower case letters indicate the matched negative control for each sample (a-g). The product size for β actin is 178 Kbases (Kb). The gel shows fluorescence of ethidium bromide stained polymerase chain reaction products resolved by electrophoresis. Bands were detected under UV light and compared to the 100 Kb pair ladder in the first lane.

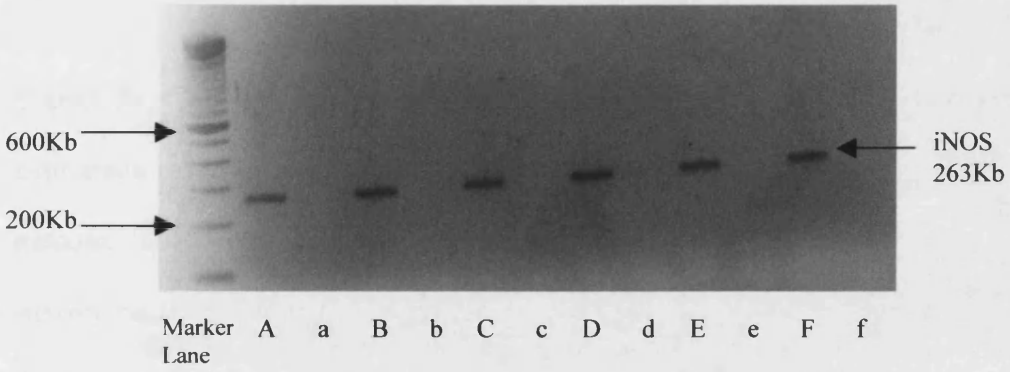


Figure 5. iNOS expression in colonic biopsies from 6 patients with ulcerative colitis (A-F). Lower case letters indicate the matched negative control for each sample (a-f). The product size for iNOS of 263 Kb. The gel shows fluorescence of ethidium bromide stained polymerase chain reaction products resolved by electrophoresis. Bands were detected under UV light and compared to the 100 Kb pair ladder in the first lane.

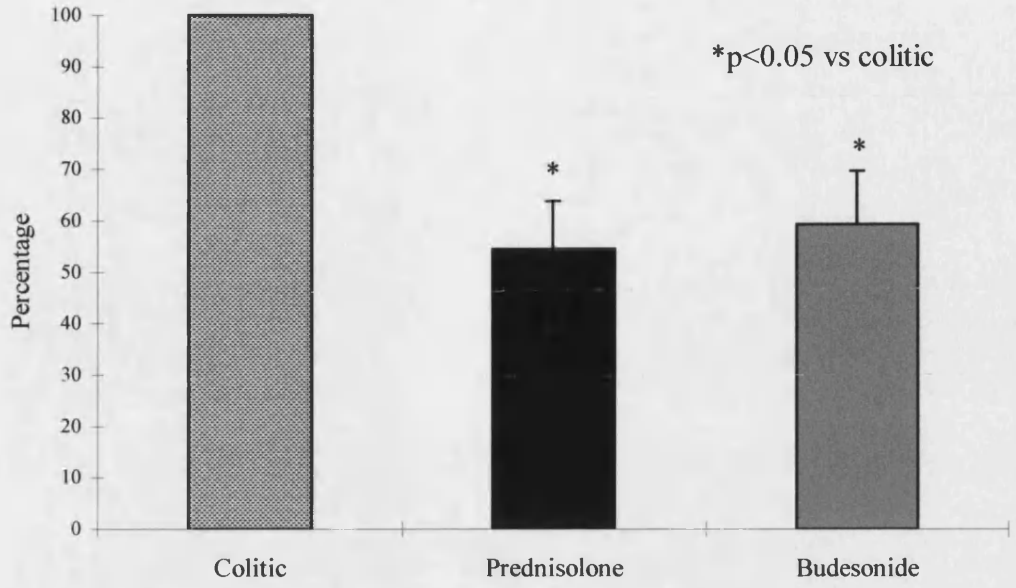


Figure 6. Mean densitometry readings (± SEM) for PCR product of iNOS expression measured as a percentage of maximum iNOS expression in the colitic mucosa. To obtain a semi-quantitative measurement of the effect of the two steroids on the biopsies, the RNA underwent PCR with the two different primers at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the two steroids. Levels for iNOS were compared to those for β Actin. This showed a significant fall in levels of iNOS when treated with either Prednisolone or Budesonide compared to levels in the untreated biopsies ($p<0.05$).

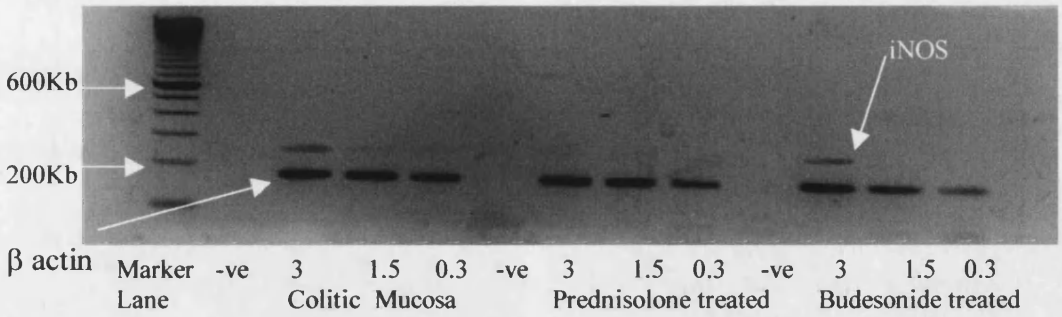


Figure 7. Representative PCR blot of three serial dilutions (3, 1.5 and 0.3 μl) of mRNA from colonic mucosa from a single colitic patient duel probed for β-actin and iNOS. Each sample also has a negative control (-ve). The product size for iNOS of 263 Kb. The product size for β actin of 176 Kb. The gel shows fluorescence of ethidium bromide stained polymerase chain reaction products resolved by electrophoresis. Bands were detected under UV light and compared to the 100 Kb pair ladder in the first lane.

3.2.2. IL-8 Activity in Colonic Biopsies From Inflamed Mucosa.

At 40 hours baseline IL-8 production per 100ug of protein in paired biopsies from inflamed areas in colitic patients (n=9) was 57.3 ± 14.8 ng/100 μg protein (mean ± SEM). With incubation with prednisolone the IL-8 production fell to 27.2 ± 9.7 ng/100μg protein ($p<0.05$) and with budesonide production fell to 17.0 ± 4.2 ng/100μg protein ($p<0.05$). (Table 2) (Figure 8)

Patient (No)	Basal	Prednisolone	Budesonide
1	46	2	10
2	55	86	35
3	158	6	18
4	85	63	19
5	25	16	13
6	64	26	14
7	56	31	40
8	14	12	1
9	13	4	7

Table 2. IL-8 production by cultures of human colonic mucosa from inflamed mucosa: Effect of incubation with 30nM of either prednisolone or budesonide. All values expressed as ng/100μg protein

Paired biopsies from inflamed areas in the colon in patients with colitis were incubated with either prednisolone or budesonide for 40 hours. At the end of this period the mRNA from the biopsies was extracted and then using primers for IL-8 underwent PCR. This clearly showed that RNA for IL-8 was present in the biopsies (Figure 9). To estimate the effect of the two steroids on the biopsies the RNA underwent PCR with two different primers at the same time: β actin and IL-8. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the

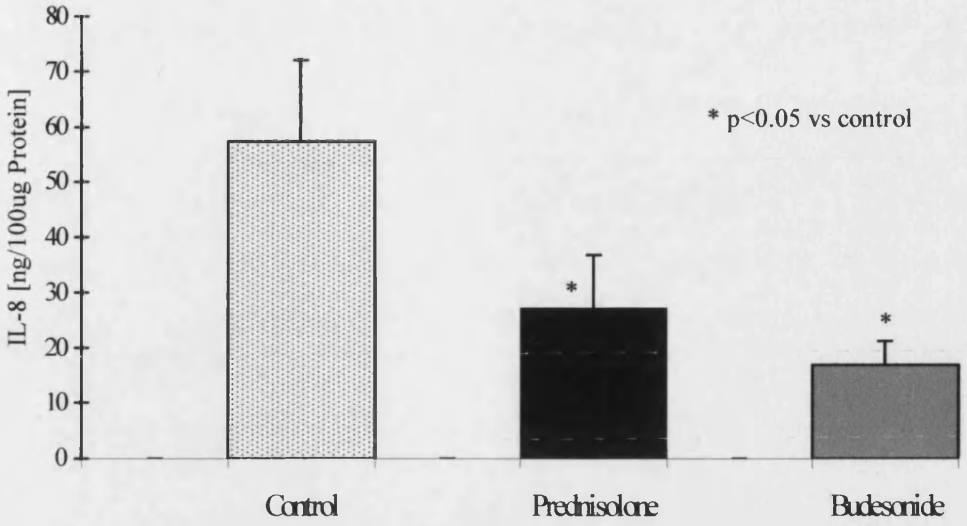


Figure 8. IL-8 production by colonic biopsies taken from inflamed areas in patients (n=9) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of IL-8 produced by the biopsies in 40 hours. Paired biopsies were also incubated with either prednisolone or budesonide at a concentration of 30nM. IL-8 levels were determined in supernatants, using an ELISA specific to IL-8. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. IL-8 levels are expressed as ng/ml in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of nine experiments. A significant effect was found using this dose of the two steroids compared to control samples ($p<0.05$), although there was no difference between the two steroids at the dose studied.

two steroids. Levels for IL-8 were compared to those for β Actin. This showed a significant fall in levels of IL-8 when treated with either Prednisolone or Budesonide compared to levels in the untreated biopsies. Levels fell to 43.2% ± 7.9% ($p<0.05$) when treated with prednisolone and 51.4% ± 3.0 % ($p<0.05$) (Figure 10). Representative blots from a patient are shown (Figure 11.)

3.2.3. iNOS Activity In Colonic Biopsies From Normal Colonic Mucosa.

To examine whether the cytokine stimulation produces NO production that is unable to be inhibited by prednisolone or budesonide, nitrite was measured in the supernatant from three cytokine stimulated colonic biopsies from patients without colitis. These were patients undergoing colonoscopy for abdominal pain or diarrhoea in which no inflammation was found. Any patients who had any inflammation reported were not included in the experiments.

Mucosal biopsy specimens of each patient were placed in four wells of a six well plate containing culture media with the appropriate stimuli per well. Two of the four wells were pre-treated for two hours with 30nM of either prednisolone or budesonide. After treatment, total protein was estimated per well and the individual nitrite content of each well was measured and expressed as pmoles/100μg Protein. (Table 3).

At 40 hours baseline mean nitrite production per 100ug of protein in non-inflamed biopsies from patients without colitis (n=12) (M:F 4:8) was 748 pmoles/100 μg protein. After stimulation with 3 cytokines the mean nitrite rose significantly to 2935 ($p<0.01$.) Pre-treatment with prednisolone at 30nM significantly decreased mean nitrite levels to 2132 pmoles/100 μg protein ($p<0.05$).

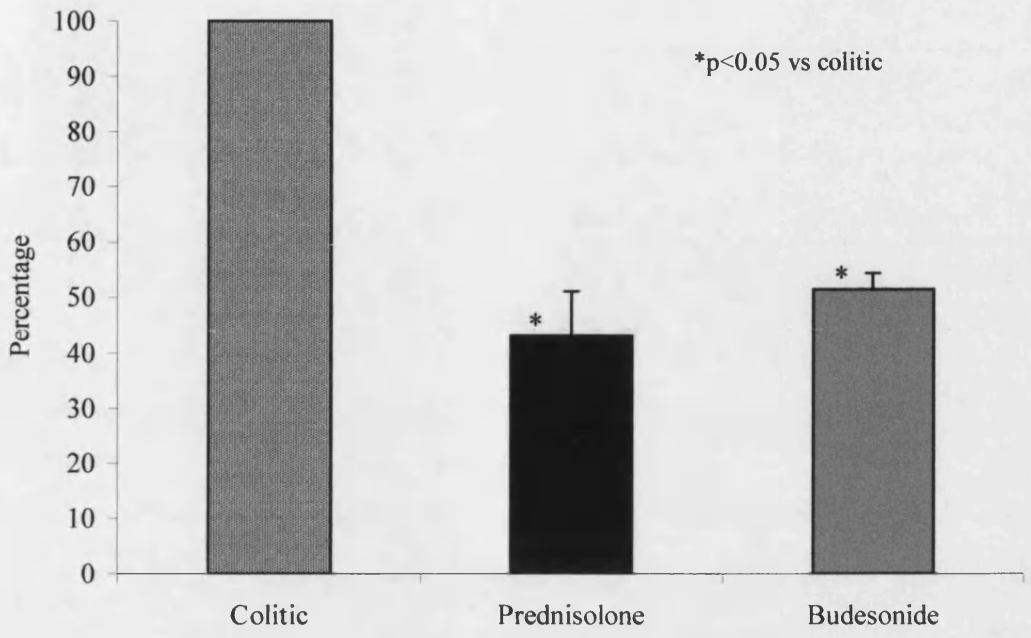


Figure 10. Mean densitometry readings (± SEM) for PCR product of IL-8 expression measured as a percentage of maximum IL-8 expression in the colitic mucosa (n=4). To obtain a semi-quantitative measurement of the effect of the two steroids on the biopsies, the RNA underwent PCR with the two different primers at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the two steroids. Levels for IL-8 were compared to those for β Actin. This showed a significant fall in levels of iNOS when treated with either Prednisolone or Budesonide compared to levels in the untreated biopsies ($p<0.05$).

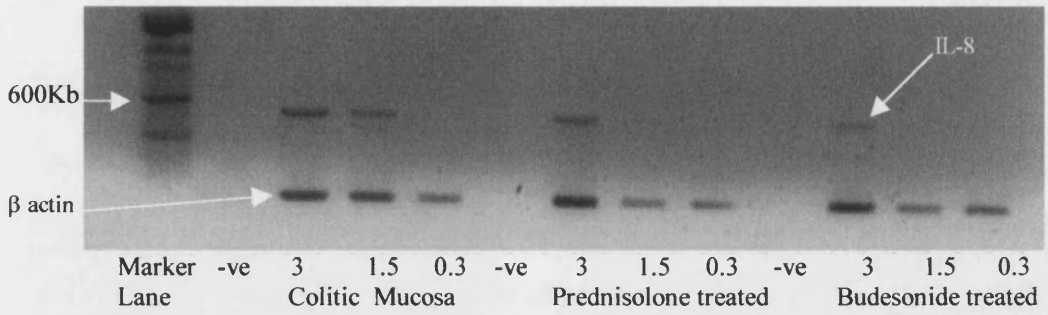


Figure 11. Representative PCR blot of three serial dilutions (3, 1.5 and 0.3 μl) of mRNA from colonic mucosa from a single colitic patient duel probed for β-actin and IL-8. Each sample also has a negative control (-ve). The product size for IL-8 of 562 Kb. The product size for β actin of 176 Kb. The gel shows fluorescence of ethidium bromide stained polymerase chain reaction products resolved by electrophoresis. Bands were detected under UV light and compared to the 100 Kb pair ladder in the first lane.

Pre-treatment with budesonide at 30nM decreased mean nitrite production to 2493 pmoles/100 μg. ($p<0.05$) (Figure12)

Patient (NO)	Basal	Cytokine stimulated (3C)	3C + Prednisolone	3C+ Budesonide
1	60	285	276	456
2	133	340	272	604
3	119	140	6	10
4	21	97	15	13
5	659	4275	3216	4081
6	374	6730	4115	4316
7	250	2196	763	605
8	126	852	895	901
9	421	1143	453	865
10	750	1130	399	362
11	667	2642	906	724
12	335	1962	938	237

Table 3. Nitrite production by cultures of human colonic mucosa from normal colon. Effect of pre-incubation with 30nM of either prednisolone or budesonide 2 hours prior to stimulation with a 3 Cytokine mixture. All values expressed as pmoles/100μg protein

Paired biopsies from histologically normal areas in the colon in patients who underwent colonoscopy for reasons other than colitis were placed in four wells of a six well plate containing culture media with the appropriate stimuli per well. Two of the four wells were pre-treated for two hours with 30nM of either prednisolone or budesonide for 40 hours. At the end of this period the mRNA from the biopsies was extracted. To estimate the effect of the two steroids on the biopsies the RNA again underwent PCR with the primers for β actin and iNOS at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the two steroids. Levels for iNOS were compared to those for β Actin.

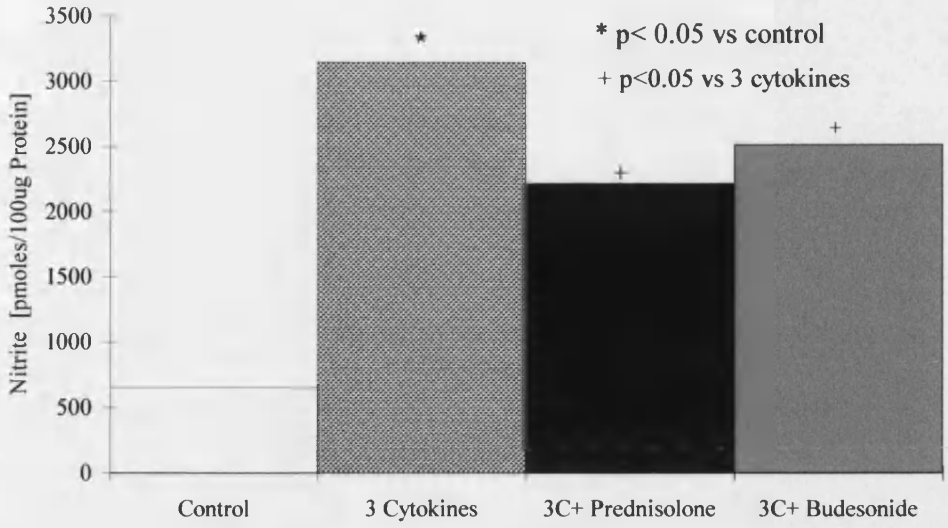


Figure 12. Nitrite production by colonic biopsies taken from histologically normal areas in patients (n=12) without colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of nitrite produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ ≠(300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were also incubated with either prednisolone or budesonide at a concentration of 30nM for two hours prior to addition of the cytokines. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. Nitrite levels are expressed as pmoles in the supernatant per 100μgrams of protein. Each bar is the mean of 12 different patients. A significant increase in nitrite production was found when treating the biopsies with the three cytokines compared to controls. ($p<0.05$). There was also a significant inhibition of cytokine stimulated nitrite production with pre-incubation of both budesonide and prednisolone ($p<0.05$).

This showed a significant rise in the levels of iNOS in the three cytokine stimulated specimen compared to the control specimen from a level of 26.3± 11.3% to 100% of maximum activity. Levels fell to 44.7% ± 13.1% ($p<0.05$) when pre-treated with prednisolone and 52.4% ± 11.3% ($p<0.05$) when pre-treated with budesonide. (Figure 13) Representative blots from a patient are shown in figure 14.

3.2.4 IL-8 Activity in Colonic Biopsies From Normal Colonic Mucosa.

Paired colonic biopsies from patients without colitis were taken at the same time and stimulated with the same cytokine mixture. Supernatant was then removed at 40 hours. At this time baseline IL-8 production per 100ug of protein in non-inflamed biopsies from patients (n=12) (M:F 4:8) was 17.0 ± 2.7 ngml/100μg protein (control ± SEM). (table 4)

Patient No	Basal	Cytokine stimulated (3C)	3C + Prednisolone	3C+ Budesonide
1	2	9	1	1
2	17	66	2	4
3	34	27	11	13
4	35	28	9	13
5	15	37	33	17
6	16	38	24	23
7	16	32	20	9
8	4	11	11	5
9	15	55	8	1
10	16	25	7	7
11	19	30	9	14
12	14	8	16	8

Table 4. IL-8 production by cultures of human colonic mucosa from normal colon. Effect of pre-incubation with 30nM of either prednisolone or budesonide 2 hours prior to stimulation with a 3 Cytokine mixture. All values expressed as ng/100μg protein

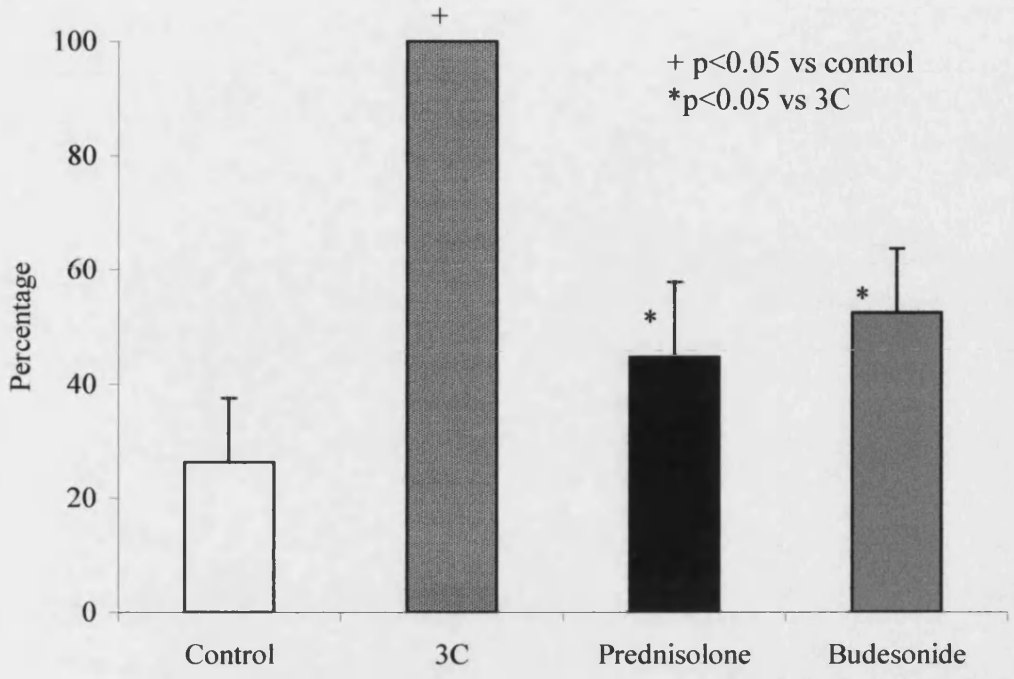
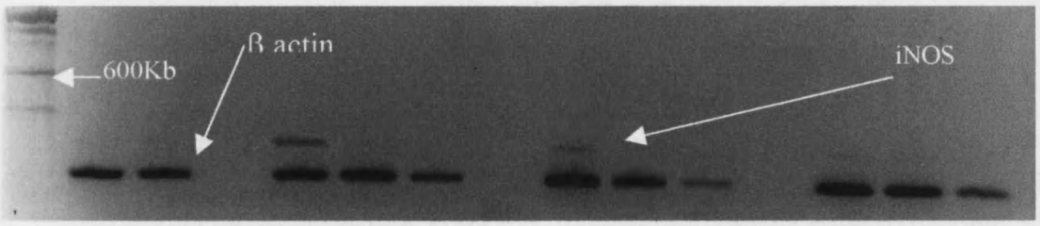


Figure 13. Mean densitometry readings (± SEM) for PCR product of iNOS expression measured as a percentage of maximum iNOS expression in cytokine stimulated colonic mucosa. Biopsies were incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were pre-incubated with either prednisolone or budesonide at a concentration of 30nM for two hours prior to addition of the cytokines. To obtain a semi-quantitative measurement of the effect of the two steroids on the biopsies, the RNA underwent PCR with the two different primers at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the two steroids. Levels for iNOS were compared to those for β Actin. This showed a significant rise in the level of iNOS from the unstimulated biopsies. There was also a fall in levels of iNOS when treated with either Prednisolone or Budesonide compared to levels in the cytokine stimulated biopsies ($p<0.05$).



Marker Control	-ve	3	1.5	0.3	-ve	3	1.5	0.3	-ve	3	1.5	0.3
Lane		3C stimulated				3C + prednisolone				3C +budesonide		

Figure 14. Representative PCR biot of three serial dilutions (3, 1.5 and 0.3 µl) of mRNA from colonic mucosa from a single subject duel probed for β-actin and iNOS. Biopsies were incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ(300U/ml) added in combination (3C). Paired samples were pre-treated with 30nM of either prednisolone or budesonide. Each sample also has a negative control (-ve). The product size for iNOS of 263 Kb. The product size for 2 actin of 176 Kb. The gel shows fluorescence of ethidium bromide stained polymerase chain reaction products resolved by electrophoresis. Bands were detected under UV light and compared to the 100 Kb pair ladder in the first lane

After stimulation with 3 cytokines this rose to 30.3 ± 5.1 $p<0.05$. Pre-treatment with prednisolone at 30nM decreased IL-8 production to 12.6 ± 2.6 ng/100 μg protein ($p<0.01$). Pre-treatment with budesonide at 30nM decreased IL-8 production to 9.5 ± 1.9 ng/100 μg protein ($p<0.01$). (Figure 15)

3.2.5 iNOS Activity In Biopsies From Histologically Normal Colonic Mucosa in Patients with Colitis

To further examine whether cytokine stimulation produces NO production that is unable to be inhibited by prednisolone or budesonide, nitrite was measured in the supernatant from three cytokine stimulated colonic biopsies from non-inflamed areas of the colon in patients with colitis. (n=4) (M:F 2:2) These were patients undergoing colonoscopy for assessment of colitis in which there were areas that were macroscopically normal. Any patients who had inflammation reported in these biopsies were not included in these experiments.

Mucosal biopsy specimens of each patient were placed in four wells of a six well plate containing culture media with the appropriate stimuli per well. Two of the four wells were pre-treated for two hours with 30nM of either prednisolone or budesonide. After treatment, total protein was estimated per well and the individual nitrite content of each well was measured and expressed as pmoles/100μg Protein. (Table 5)

At 40 hours baseline nitrite production per 100μg of protein in non-inflamed biopsies from patients with colitis was 497 ± 424 pmoles/100 μg protein (control ± SEM). After stimulation with 3 cytokines this rose to 2219 ± 1074 pmoles/100 μg protein ($p<0.05$). Pre-treatment with prednisolone at 30nM significantly decrease nitrite production which fell to 202 ± 130 pmoles/100 μg protein

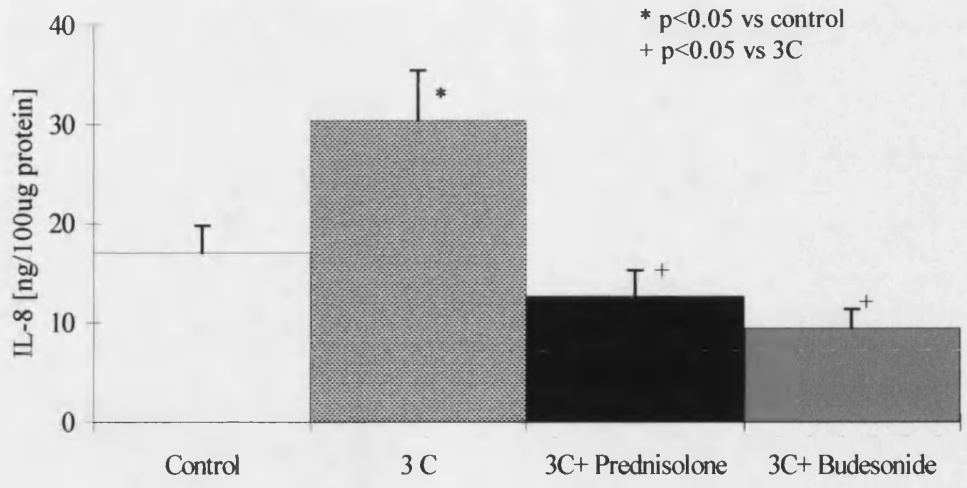


Figure 15. IL-8 production by colonic biopsies taken from histologically normal areas in patients (n=12) without colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of nitrite produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Paired biopsies pre-treated with either prednisolone or budesonide at a concentration of 30nM for two hours were also incubated with the three cytokines. IL-8 levels were determined in supernatants, using an ELISA specific to IL-8. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. IL-8 levels are expressed as ng/ml in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of 12 different patients. A significant increase in IL-8 production was found when treating the biopsies with the three cytokines compared to controls ($p<0.05$). A significant inhibition of this effect using the dose of either the two steroids studied compared to the cytokine stimulated samples was also noted ($p<0.05$).

($p<0.01$). Pre-treatment with budesonide at 30nM significantly decreased nitrite production to 260 ± 54 pmoles/100 μg. ($p<0.01$) (Figure 16)

Patient No	Basal	Cytokine stimulated (3C)	3C + Prednisolone	3C+ Budesonide
1	920	3294	332	314
2	73	1145	72	54
3	238	1972	162	290
4	758	2466	244	228

Table 5. Nitrite production by cultures of macroscopically normal human colonic mucosa from patients with colitis. Effect of pre-incubation with 30nM of either prednisolone or budesonide 2 hours prior to stimulation with a 3 Cytokine mixture. All values expressed as pmoles/100μg protein

3.2.6.IL-8 Activity In Biopsies From Histologically Normal Colonic Mucosa in Patients with Colitis

At 40 hours baseline IL-8 production per 100μg of protein in non-inflamed biopsies from patients with colitis was 12.6 ± 1.8 ng/100 μg protein (control ± SEM). After stimulation with 3 cytokines this rose to 32.0 ± 7.3 ng/100μg protein ($p<0.05$). Pre-treatment with prednisolone at 30nM significantly decreased IL-8 production which fell to 7.4 ± 2.9 ng/100 μg protein ($p<0.01$).

Patient No	Basal	Cytokine stimulated (3C)	3C + Prednisolone	3C+ Budesonide
1	13	20	3	4
2	11	36	6	9
3	10	28	8	6
4	16	44	13	17

Table 6. IL-8 production by cultures of macroscopically normal human colonic mucosa from patients with colitis. Effect of pre-incubation with 30nM of either prednisolone or budesonide 2 hours prior to stimulation with a 3 Cytokine mixture. All values expressed as ng/100μg protein.

Pre-treatment with budesonide at 30nM significantly decreased IL-8 production to 9.0 ± 4 ng/100 μg. (Table 6)(Figure 17)

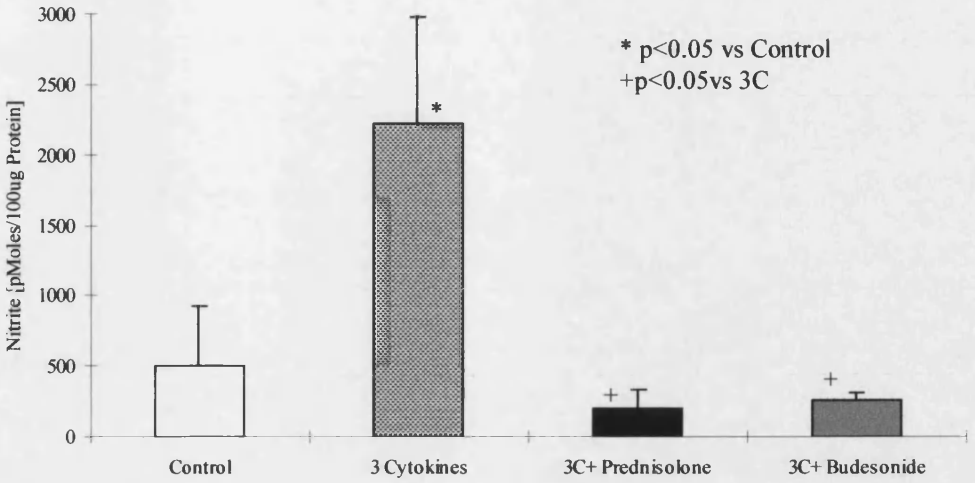


Figure 16. Nitrite production by colonic biopsies taken from histologically normal areas in patients (n=4) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of nitrite produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were also incubated with either prednisolone or budesonide at a concentration of 30nM for two hours prior to addition of the cytokines. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. Nitrite levels are expressed as pmoles in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of 4 different patients. A significant increase in nitrite production was found when treating the biopsies with the three cytokines compared to controls ($p<0.05$). A significant inhibitory effect of pre-treatment with either of the two steroids of the cytokine stimulated samples was noted ($p<0.05$). There was no significant difference between the two steroids at the dose studied.

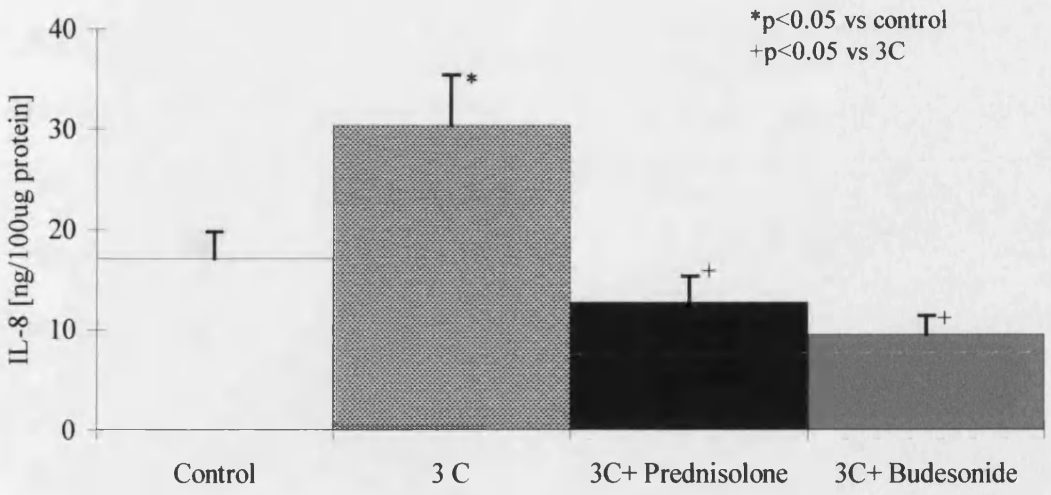


Figure 17. IL-8 production by colonic biopsies taken from histologically normal areas in patients (n=4) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of IL-8 produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were also incubated with either prednisolone or budesonide at a concentration of 30nM for two hours prior to addition of the cytokines. IL-8 levels were determined in supernatants, using an ELISA specific to IL-8. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. IL-8 levels are expressed as ng/ml in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of 4 different patients. A significant increase in IL-8 production was found when treating the biopsies with the three cytokines compared to controls ($p<0.05$). A significant inhibitory effect of pre-treatment with either of the two steroids of the cytokine stimulated samples was noted ($p<0.05$). There was no significant difference between the two steroids at the dose studied.

3.3 Discussion and Technical Points

In the colonic epithelial cell line model (HT-29) the production of both IL-8 and NO as measured by nitrite are essentially unaffected by a wide dose of steroids ranging from 10nM to 300mM. Any reduction in NO production and IL-8 production only occurs at doses that far exceed any concentrations that could be achieved in treatment doses, for example 100μM or above. This is in contrast to the experience of workers who have used steroids on other cell types and found that iNOS transcription can be inhibited. In these studies workers have shown that in some cell types e.g.Caco-2 and RAW 264.7 cells that transcription of iNOS can be inhibited by incubation with steroids (Walker *et al.*, 1997; Cavicchi and Whittle, 1999). The evidence to date suggests that both IL-8 and NO are important in the pathophysiology of inflammatory bowel disease and as we know that both prednisolone and budesonide are important clinically in the management of colitis then another explanation appeared likely. There were several possibilities. Firstly, was NO or IL-8 production by colonic epithelial cells unaffected by steroids? Secondly, was the method of stimulating HT-29 cells with IL-1α, IFN-γ and TNF-α to produce NO and IL-8, effectively steroid resistant? Thirdly, was there another cell type that acted directly upon the colonic epithelial cell which was sensitive to steroids? Lastly, was the HT-29 cell so different to the normal colonic epithelial cell that it was immune to the effect of steroids?

The results of the experiment looking at biopsies from patients with active inflammation show clearly that NO production and IL-8 production is dramatically reduced when incubated with very low doses of both the steroids studied (30nM) i.e. 1000 times less dose than that which had an effect on the cytokine stimulated HT-29 cells. This would suggest that the evidence that

steroids when used for the management of IBD reduces inflammation at least in part by reducing IL-8 and nitrite production. One of the interesting differences in this study compared with other studies is that all of these patients are newly diagnosed colitic patients who are on no treatment prior to the experiment. Other studies looking at immunohistochemistry and other changes in NO biochemistry have used patients who are already well established on treatment often including steroids.

This effect of the steroids was also confirmed by the data from the PCR results looking at iNOS and IL-8 mRNA. This first experiment shows that mRNA for iNOS is produced in colitis and that the levels of the mRNA are reduced when the inflamed biopsies were incubated with either prednisolone or budesonide. Although the method used of dual probing is only semi-quantitative the evidence combined with the nitrite and IL-8 data from the incubated biopsies from inflamed colonic biopsies would strongly suggest that this is a real reduction in iNOS and IL-8 production by down regulation of mRNA. Ideally, northern blot techniques would have been used, and in fact that this was tried on several occasions. Unfortunately, even after 20 hours of incubation too much degradation of the mRNA had occurred and this did not allow us to proceed with this method of examining how much mRNA was present. Also results from Western blot analysis would have been presented to confirm a lowering of the product of the iNOS gene. Unfortunately within the time restraints allowed to do the work in, I was unable to do this.

As the response of histologically normal biopsies to the same cytokine stimulation used in the HT-29 cell model is markedly different, a further set of experiments was performed. Three specific queries were addressed. Firstly, whether the

cytokines were able to stimulate the biopsies to produce nitric oxide via stimulation of iNOS? Secondly, whether the cytokines were creating conditions where steroids were unable to act upon the epithelial cells? We know from the previous work from Singer and Kolios that iNOS is almost exclusively produced in colitis by colonic epithelial cells. Therefore any changes in iNOS are thought to be due to effects on the colonic epithelium. Thirdly was there any difference in responses between histologically normal biopsies in patients with or without colitis?

These experiments showed that normal mucosa when exposed to IL-1α, TNF-α and IFN-γ produces large quantities of both IL-8 and NO as measured by nitrite. In both colitic and non-colitic patients, both nitrite and IL-8 are suppressed in histologically normal when pre-treated with steroids prior to stimulation. The levels of iNOS mRNA as measured by PCR does indeed fall when pre-treated with either one of the two steroids prior to stimulation with the three cytokines. This suggests that teanscription of iNOS mRNA is inhibited by the steroids and that this is in part at least the mechanism in reduction of nitrite levels. This similarity between inflamed mucosa and stimulated mucosa would suggest that the site of action of the steroids is similar, and that the steroids are able to prevent the stimulatory factors acting upon the colonic epithelial cells. It is not clear whether the stimulatory cytokines act directly upon the epithelial cell to induce the transciption of iNOS, but it is likely that they act here and upon other cells in the colonic mucosa e.g. Intraepithelial lymphocytes. The effect of the steroids is not as marked as in the inflamed mucosa alone but this may be due to a variety of factors such as maximal stimulation in the cytokine model which may not be the case in the inflamed mucosa or that the combination of cytokines used to

stimulate the normal mucosa somehow induces transcription in a way that is harder to inhibit.

Chapter 4: RESULTS

Effects Of IL-4, IL-10 and IL-13 on Inducible Nitric Oxide Expression in Colonic Epithelial Cells

4.1 Effect Of Incubation With IL-4, IL-10 Or IL-13 On Inducible Nitric Oxide Synthase (iNOS) And IL-8 Activity In Colonic Biopsies

4.1.1. iNOS Activity In Colonic Biopsies From Inflamed Mucosa.

To examine the effects of the three T cell derived cytokines IL-4, IL-10 and IL-13 have on colonic biopsies a series of experiments were designed. The effect of IL-4, IL-10 or IL-13 has on NO and IL-8 production by colonic biopsies was examined in paired colonic biopsies from patients with colitis. These were patients undergoing colonoscopy in which a new diagnosis of colitis was found. Only patients who had inflammation consistent with ulcerative colitis reported were included in the experiments.

Mucosal biopsy specimens of each patient were placed in five wells of a six well plate containing culture media with the appropriate stimuli per well. Three of the five wells were treated with 30ng/ml of IL-4, IL-10 or IL-13. After 40 hours total protein was estimated per well and the individual nitrite content of each well was measured and expressed as pmoles/100μg Protein. (Table 7)

At 40 hours baseline mean nitrite production per 100ug of protein in biopsies from inflamed areas in colitic patients (n=8)(M:F 6:3) was 1427 pmoles/100 μg protein. Incubation with IL-10 mean nitrite levels fell to 475 pmoles/100μg protein ($p<0.05$). Incubation with IL-4 mean nitrite levels fell to 901 which was

non-significant and with IL-13 mean nitrite was measured at 1433 pmoles/100μg protein. (Figure 18)

Patient No	Basal	IL-4	IL-10	IL-13
1	1410	2692	138	682
2	1346	408	450	224
3	1586	744	556	684
4	1016	480	280	348
5	342	738	112	1146
6	1882	1174	454	6600
7	1240	262	208	354
8	2600	710	1603	1426

Table 7. Nitrite production by cultures of human colonic mucosa from inflamed mucosa. Effect of incubation with 30ng/ml of IL-4, IL-10 or IL-13. All values expressed as pmoles/100μg protein

Further paired biopsies from inflamed areas in the colon in patients with colitis were incubated with IL-4, IL-10 or IL-13 for 40 hours. At the end of this period the mRNA from the biopsies was extracted and then the mRNA underwent PCR using primers for iNOS and β actin. To estimate the effect of the anti-inflammatory cytokines on the biopsies, the RNA underwent PCR with the two different primers at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the two IL-4, IL-10 or IL-13. Levels for iNOS were compared to those for β Actin. This showed a significant fall in levels of iNOS when treated with

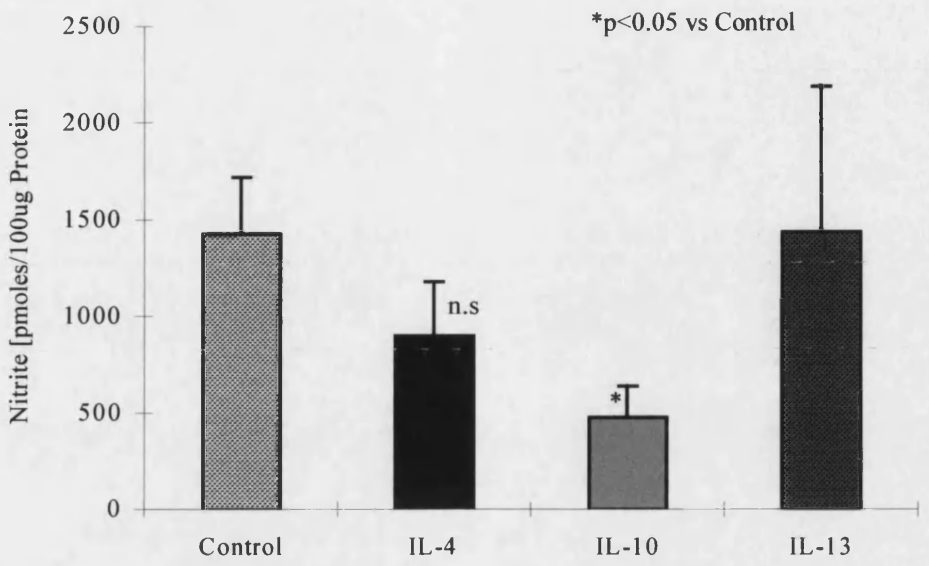


Figure 18. Nitrite production by colonic biopsies taken from inflamed areas in patients (n=8) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of nitrite produced by the biopsies in 40 hours. Paired biopsies were also incubated with IL-4, IL-10 or IL-13 at a concentration of 30ng/ml. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. Nitrite levels are expressed as pmoles in the supernatant per 100μgrams of protein. Each bar is the mean of eight experiments. A significant effect was found using this dose of IL-10 compared to control samples ($p<0.05$). There was no significant difference between either IL-4 or IL-13 and the control biopsies studied.

either IL-4, IL-10 or IL-13 compared to levels in the untreated biopsies. Levels fell to 60.3% ± 2.0% ($p<0.05$) when treated with IL-4 ($p<0.05$), 25.1 ± 8.3% when treated with IL-10 ($p<0.01$) and 78.2% ± 16.8% when treated with IL-13 (not significant) (figure 19). Representative blots from a patient are shown (figure 20).

4.1.2. IL-8 Activity In Colonic Biopsies From Inflamed Mucosa

At 40 hours baseline IL-8 production per 100ug of protein in biopsies from inflamed areas in colitic patients was 64 ± 9 ng/100 μg protein (control ± SEM). With incubation with IL-4 this fell to 27 ± 6 ng/100 μg protein ($p<0.05$) and with IL-10 this fell to 35 ± 7 ng/100μg protein ($p<0.05$). With incubation with IL-13 there was no significant difference with IL-8 levels of 64 ± 28 ng/100μg protein (Table 8) (Figure 21)

Patient (No)	Basal	IL-4	IL-10	IL-13
1	105	48	47	42
2	34	14	13	37
3	59	34	16	18
4	87	8	60	8
5	73	56	55	38
6	34	15	7	46
7	46	28	45	255
8	78	14	36	71

Table 8. IL-8 production by cultures of human colonic mucosa from inflamed mucosa: Effect of incubation with 30ng/ml of IL-4, IL-10 or IL-13. All values expressed as ng/100μg protein

Further paired biopsies from inflamed areas in the colon in patients with colitis were incubated with IL-4, IL-10 or IL-13 for 40 hours. At the end of this period the

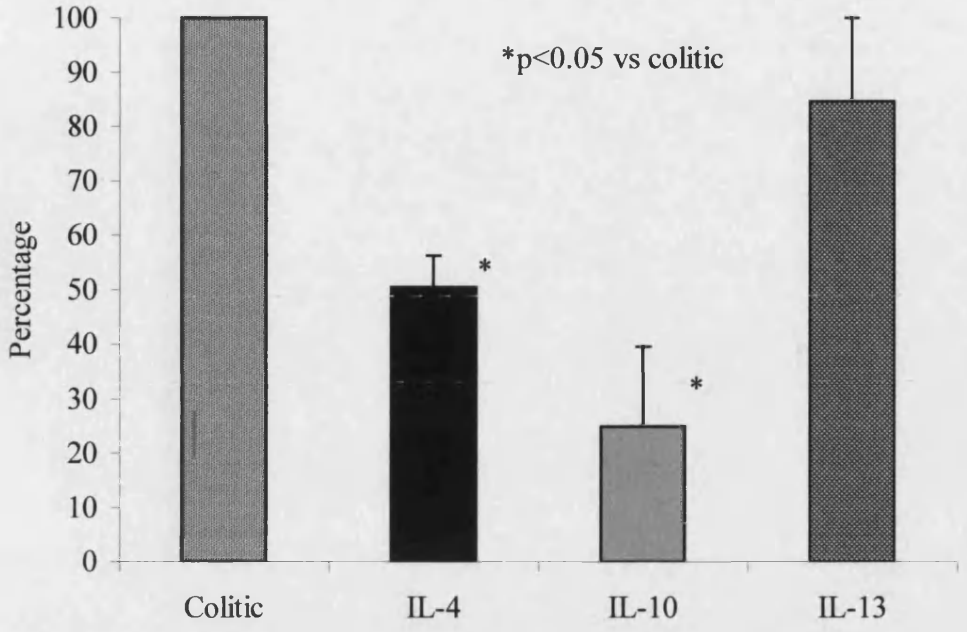


Figure 19. Mean densitometry readings (± SEM) for PCR product of iNOS expression measured as a percentage of maximum iNOS expression in the cytokine stimulated (3C) colonic mucosa (n=4). To obtain a semi-quantitative measurement of the effect of the cytokines on the biopsies, the RNA underwent PCR with the two different primers at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the cytokines. Levels for iNOS were compared to those for β Actin. There was a significant rise in the levels of iNOS mRNA in the cytokine stimulated mucosa compared to the unstimulated normal mucosa ($p<0.05$). There was a significant fall in levels of iNOS when treated with IL-4 or IL-10 to levels in the untreated colitic mucosa ($p<0.05$). No significant reduction with IL-13 was found at the dose studied.

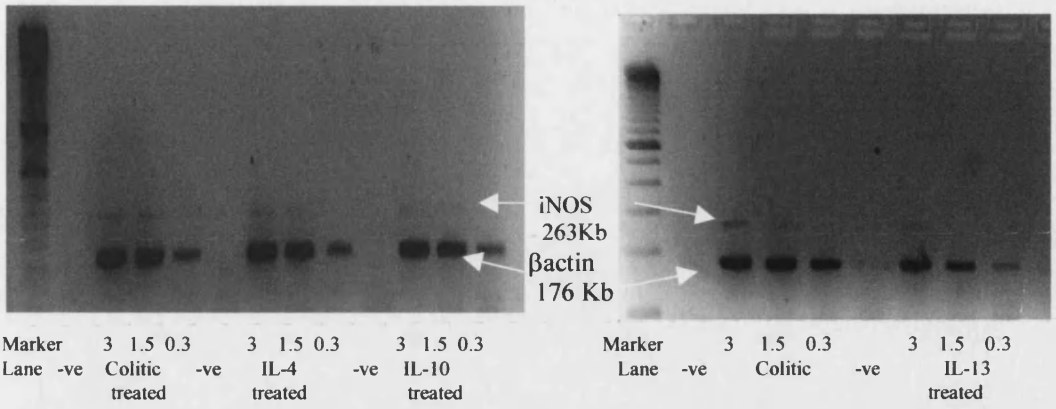


Figure 20. Representative PCR blot of three serial dilutions (3, 1.5 and 0.3 μl) of mRNA from colonic mucosa from a single colitic patient duel probed for β-actin and iNOS. Each sample also has a negative control (-ve). The product size for iNOS of 263 Kb. The product size for 2 actin of 176 Kb. The gel shows fluorescence of ethidium bromide stained polymerase chain reaction products resolved by electrophoresis. Bands were detected under UV light and compared to the 100 Kb pair ladder in the first lane.

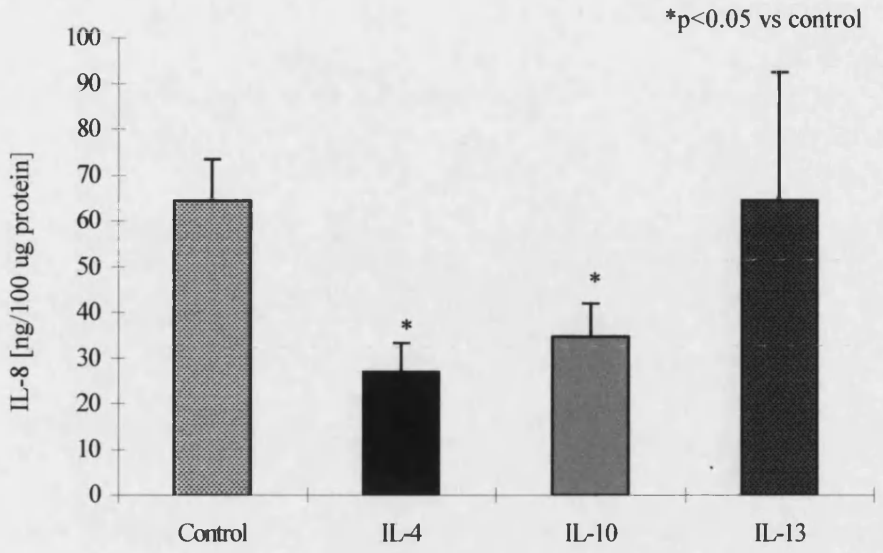


Figure 21. IL-8 production by colonic biopsies taken from inflamed areas in patients (n=8) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of IL-8 produced by the biopsies in 40 hours. Paired biopsies were also incubated with IL-4, IL-10 or IL-13 at a concentration of 30ng/ml. IL-8 levels were determined in supernatants, using an ELISA specific to IL-8. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. IL-8 levels are expressed as ng/ml in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of eight experiments. A significant effect was found using this dose of both IL-4 and IL-10 compared to control samples ($p<0.05$). There was no significant difference between IL-13 and the control biopsies. There was no significant difference between IL-4 and IL-10 at the dose studied.

mRNA from the biopsies was extracted and then using primers for IL-8 and β actin underwent PCR. To estimate the effect of the T-cell derived cytokines on the biopsies the RNA underwent PCR with the two different primers at the same time. This allowed us to obtain a semi-quantitative measure of the effect of incubation with the IL-4, IL-10 or IL-13. Levels for IL-8 were compared to those for β Actin. This showed a significant fall in levels of IL-8 when treated with either IL-4, IL-10 or IL-13 compared to levels in the untreated biopsies. Levels fell to 50.4% ± 5.7% ($p<0.05$) when treated with IL-4 ($p<0.05$), 24.9 ± 14.7% when treated with IL-10 ($p<0.01$) and 75.1% ± 21.6% when treated with IL-13 (not significant) (Figure 22). Representative blots from a patient are shown (Figure 23)

4.1.3 iNOS Activity In Colonic Biopsies From Normal Colonic Mucosa.

To examine whether the cytokine stimulation in the HT-29 cells induces NO production that is unable to be inhibited by IL-10, nitrite was measured in the supernatant from three cytokine stimulated colonic biopsies from patients without colitis. These were patients undergoing colonoscopy for abdominal pain or diarrhoea in which no inflammation was found. Any patients who had any inflammation reported in the endoscopically normal biopsies were not included in the experiments.

Mucosal biopsy specimens of each patient were placed in five wells of a six well plate containing culture media with the appropriate stimuli per well. Each of the five wells were pre-treated for two hours with 30ng/ml of either IL-4, IL-10 or IL-13. After treatment, total protein was estimated per well and the individual nitrite content of each well was measured and expressed as pmoles/100μg Protein. (Table 9).

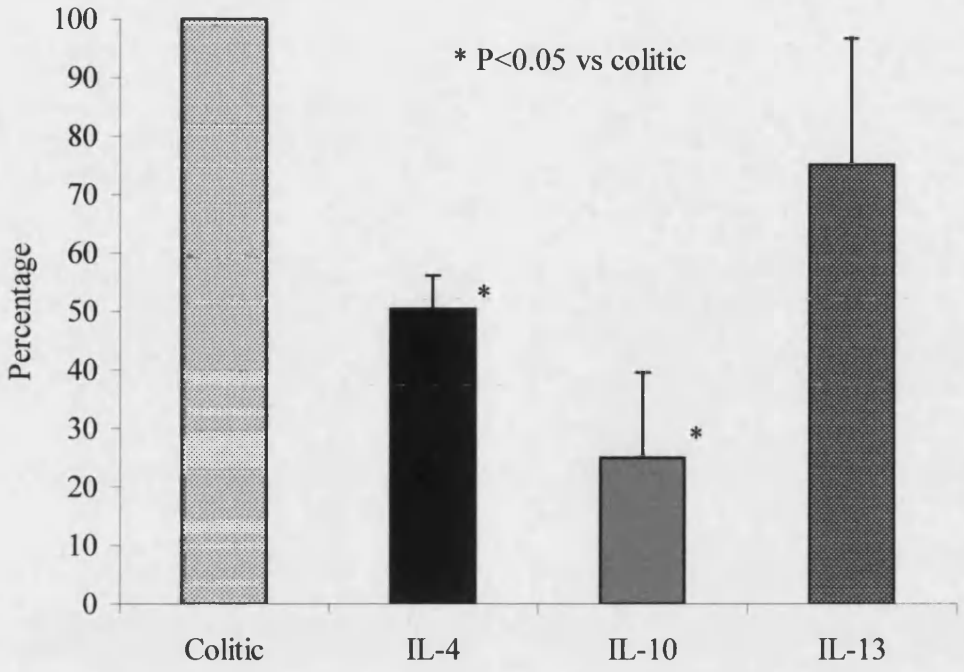


Figure 22. Mean densitometry readings (± SEM) for PCR product of IL-8 expression measured as a percentage of maximum IL-8 expression in the colitic mucosa. To obtain a semi-quantitative measurement of the effect of the cytokines on the biopsies, the RNA underwent PCR with the two different primers at the same time. This allowed a semi-quantitative measure of the effect of incubation with the cytokines studied. Levels for IL-8 were compared to those for β Actin. This showed a significant fall in levels of iNOS when treated with either IL-4 or IL-10 compared to levels in the untreated biopsies ($p<0.05$). There was no effect found of IL-13 at the dose studied.

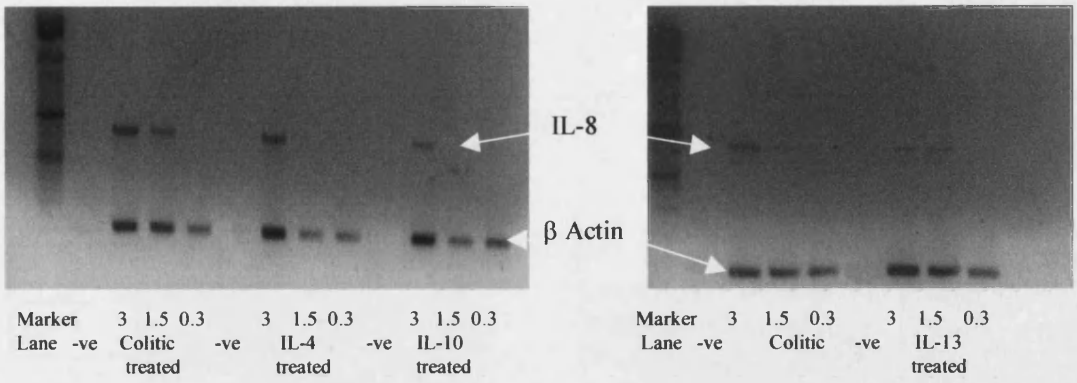


Figure 23. Representative PCR blot of three serial dilutions (3, 1.5 and 0.3 μl) of mRNA from colonic mucosa from a single colitic patient duel probed for β-actin and IL-8. Each sample also has a negative control (-ve). The product size for IL-8 of 562 Kb. The product size for 2 actin of 176 Kb. The gel shows fluorescence of ethidium bromide stained polymerase chain reaction products resolved by electrophoresis. Bands were detected under UV light and compared to the 100 Kb pair ladder in the first lane.

Patient No	Basal	Cytokine Stimulated	IL-4	IL-10	IL-13
1	277	944	191	23	99
2	65	395	117	99	50
3	472	934	19	12	18
4	136	283	69	11	132
5	470	1642	432	636	754
6	494	1487	843	765	225
7	303	699	115	497	1574
8	376	545	366	297	154
9	468	947	348	385	248
10	16	169	13	15	30

Table 9. Nitrite production by cultures of human colonic mucosa from normal colon. Effect of pre-incubation with 30ng/ml of IL-4, IL-10 or IL-13 2 hours prior to stimulation with a 3 Cytokine mixture. All values expressed as pmoles/100μg protein

At 40 hours baseline nitrite production per 100ug of protein in non-inflamed biopsies from patients without colitis was 615 ± 114 pmoles/100 μg protein (control ± SEM). After stimulation with 3 cytokines this rose to 1609 ± 308 ($p<0.05$). Pre-treatment with all three cytokines at 30 ng/ml produced a significant fall in nitrite production. IL-4 to 502 ± 162 pmoles/100 μg protein ($p<0.01$). IL-10 to 548 ± 180 ($p<0.05$), and IL-13 to 656 ± 308 ($p<0.05$). (Figure 24)

4.1.4 IL-8 Activity In Colonic Biopsies From Normal Colonic Mucosa.

Paired colonic biopsies from patients without colitis were taken at the same time and stimulated with the same cytokine mixture. Supernatant was then removed at 40 hours.

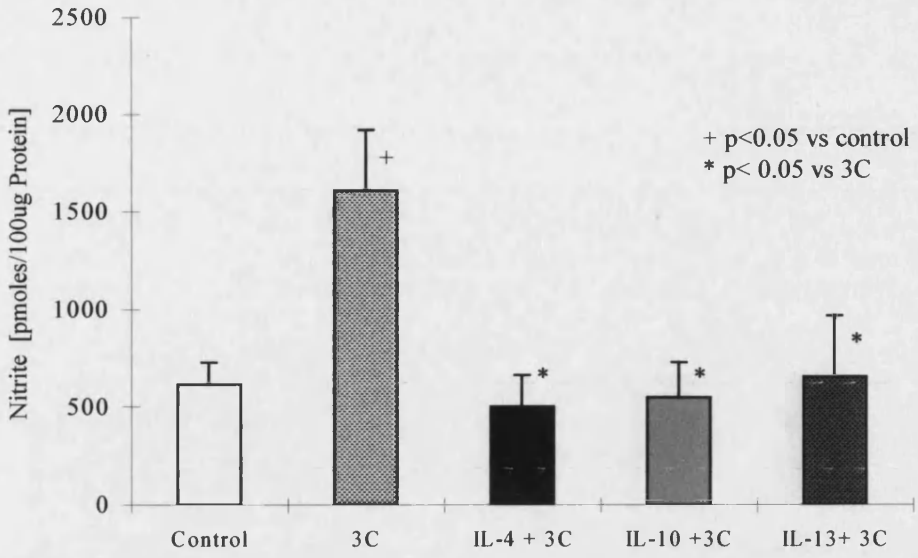


Figure 24. Nitrite production by colonic biopsies taken from histologically normal areas in patients (n=8) without colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of nitrite produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ ≠(300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were also incubated with IL-4, IL-10 or IL-13 at a concentration of 30ng/ml for two hours prior to addition of the cytokines. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. Nitrite levels are expressed as pmoles in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of eight different patients. A significant increase in nitrite production was found when treating the biopsies with the three cytokines compared to controls. ($p<0.05$). A significant inhibition of this effect using the dose of all three anti-inflammatory cytokines studied compared to the cytokine stimulated samples was noted ($p< 0.05$). There was no significant difference between the cytokines studied.

Patient No	Basal	Cytokine Stimulated (3C)	3C+IL-4	3C+IL-10	3C+IL-13
1	15	25	13	10	12
2	5	13	9	11	13
3	4	7	1	1	1
4	3	10	9	7	7
5	4	37	6	16	23
6	22	46	35	15	12
7	18	58	26	24	80
8	32	48	18	2	14
9	3	17	3	2	2
10	17	94	33	54	110

Table 10. IL-8 production by cultures of human colonic mucosa from normal colon. Effect of pre-incubation with 30ng/ml of Il-4, IL-10 or IL-13 2 hours prior to stimulation with a 3 Cytokine mixture. All values expressed as ng/100μg protein

At 40 hours baseline IL-8 production per 100ug of protein in non-inflamed biopsies from patients without colitis was 12 ± 3.2 ng/100 μg protein (control ± SEM). After stimulation with 3 cytokines this rose to 36 ± 8.6 ng/100μg protein $p<0.05$. Pre-treatment with IL-4 produced a significant fall in IL-8 production to 15 ± 3.5 ng/100 μg protein ($p<0.05$) and IL-10 led to a fall to 14 ± 4.9 ng/100μg protein ($p<0.05$). Pre-treatment with IL-13 did not lead to a significant fall 27 ± 11.6 ng/100μg protein (not significant). (Table 10)(Figure 25)

4.1.5 iNOS Activity In Biopsies From Histologically Normal Colonic Mucosa in Patients with Colitis

To further examine the effect of IL-4, IL-10 and IL-13 on cytokine stimulated NO production, nitrite was measured in the supernatant from three cytokine stimulated

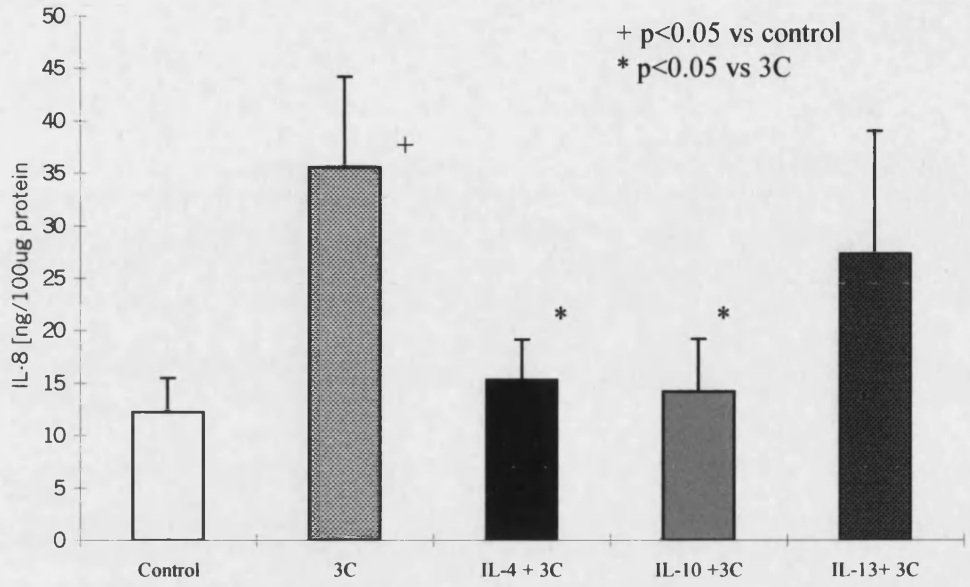


Figure 25. IL-8 production by colonic biopsies taken from histologically normal areas in patients (n=8) without colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of IL-8 produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were also incubated with IL-4, IL-10 or IL-13 at a concentration of 30ng/ml for two hours prior to addition of the cytokines. IL-8 levels were determined in supernatants, using an ELISA specific to IL-8. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. IL-8 levels are expressed as ng/ml in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of eight experiments. A significant effect was found using this dose of both IL-4 and IL-10 compared to samples stimulated with the 3C ($p<0.05$). There was no significant difference between IL-13 and the control biopsies studied. There was no significant difference between IL-4 and IL-10.

colonic biopsies from non-inflamed areas of the colon in patients with colitis. (n=4) (M:F 2:2) These were patients undergoing colonoscopy for assessment of colitis in which there were areas that were macroscopically normal. Any patients who had inflammation reported in these biopsies were not included in these experiments.

Mucosal biopsy specimens of each patient were placed in five wells of a six well plate containing culture media with the appropriate stimuli per well. Three of the five wells were pre-treated for two hours with 30ng/ml of IL-4, IL-10 or IL-13. The three pro-inflammatory cytokines were then added as in the concentrations known to induce iNOS in HT-29 cells. After treatment, total protein was estimated per well and the individual nitrite content of each well was measured and expressed as pmoles/100μg Protein. (Table 11)

At 40 hours baseline nitrite production per 100μg of protein in non-inflamed biopsies from patients with colitis was 405 ± 248 pmoles/100μg protein (control ± SEM). After stimulation with 3 cytokines this rose to 2139 ± 908 $p<0.05$. Pre-treatment with IL-4 at 30ng/ml significantly decreased nitrite production to 422 ± 207 pmoles/100μg protein $p<0.05$. Pre-treatment with IL-10 at 30ng/ml decreased nitrite production to 227 ± 138 pmoles/100μg ($p<0.05$). Pre-treatment with IL-13 at 30ng/ml had no significant decrease in nitrite production which fell to 1870 ± 1084 pmoles/100 μg. (Figure 26)

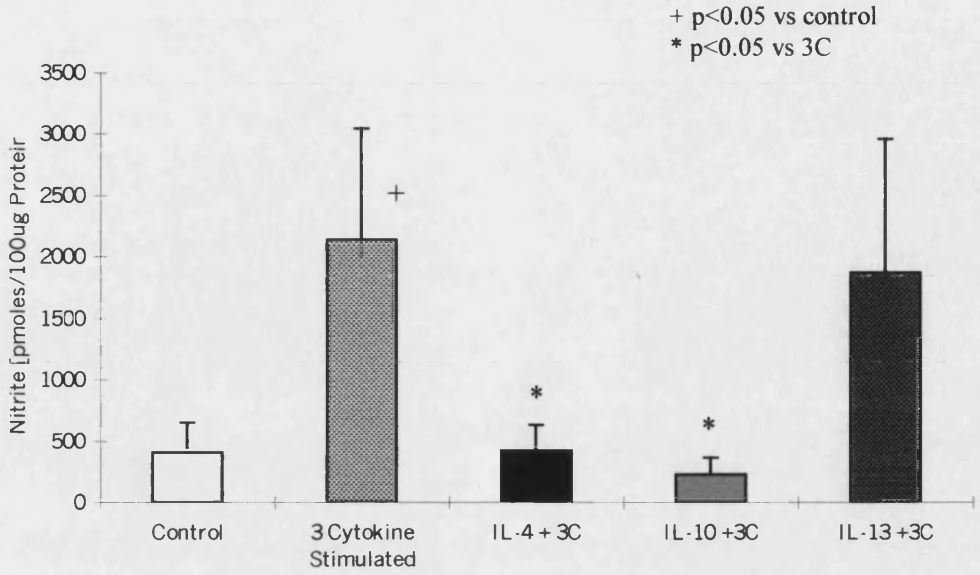


Figure 26. Nitrite production by colonic biopsies taken from histologically normal areas in patients (n=4) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of nitrite produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were also incubated with IL-4, IL-10 or IL-13 at a concentration of 30ng/ml for two hours prior to addition of the cytokines. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. Nitrite levels are expressed as pmoles in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of 4 different patients. A significant increase in nitrite production was found when treating the biopsies with the three cytokines compared to controls ($p<0.05$). A significant inhibition of stimulated nitrite production by both IL-4 and IL-10 was noted compared to the cytokine stimulated samples alone.

Patient No	Basal	Cytokine Stimulation (3C)	3C + IL-4	3C + IL-10	3C + IL-13
1	110	506	162	57	73
2	23	1129	135	29	1429
3	105	311	36	47	8
4	571	2327	511	320	2231

Table 11. Nitrite production by cultures of macroscopically normal human colonic mucosa from patients with colitis. Effect of pre-incubation with 30ng/ml of either IL-4, IL-10 or IL-13 2 hours prior to stimulation with a 3 Cytokine mixture. All values expressed as pmoles/100μg protein

4.1.6 IL-8 Activity In Biopsies From Histologically Normal Colonic Mucosa in Patients with Colitis

At 40 hours baseline IL-8 production per 100μg of protein in non-inflamed biopsies from patients with colitis was 9.1 ± 1.3 ng/100 μg protein (control ± SEM). After stimulation with 3 cytokines this rose to 19.0 ± 3.5 ng/100μg protein $p<0.05$.

Patient No	Basal	Cytokine Stimulation (3C)	3C +IL-4	3C +IL-10	3C +IL-13
1	6	14	9	7	11
2	9	19	12	10	51
3	9	15	4	1	7
4	12	29	17	17	21

Table 12. IL-8 production by cultures of macroscopically normal human colonic mucosa from patients with colitis. Effect of pre-incubation with 30ng/ml of either IL-4, IL-10 or IL-13 two hours prior to stimulation with a 3 Cytokine mixture. All values expressed as ng/100μg protein.

Pre-treatment with IL-4 at 30ng/ml significantly decreased IL-8 production that fell to 10.9 ± 2.6 ng/100 μg protein $p<0.01$. Pre-treatment with IL-10 at 30ng/ml

decreased IL-8 production to 8.0 ± 3.2ng/100 μg protein ($p<0.05$). Pre-treatment with IL-13 at 30ng/ml had no significant effect on IL-8 production that rose to 23.1 ± 9.9 ng/ 100μg protein. (Table 12)(Figure 27)

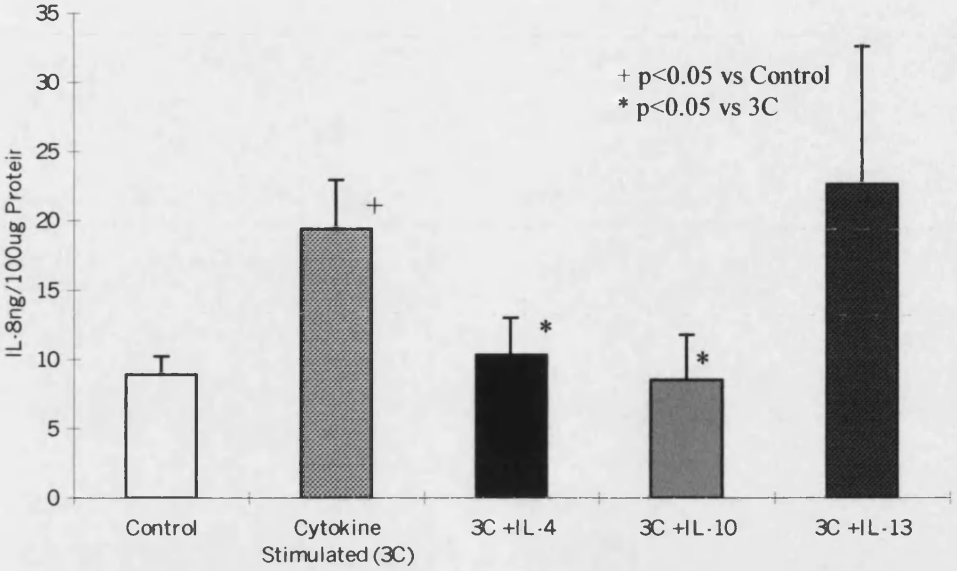


Figure 27. IL-8 production by colonic biopsies taken from histologically normal areas in patients (n=4) with colitis. The biopsies were incubated at 37° C for 40 hours. Control is the amount of IL-8 produced by the biopsies in 40 hours. Paired biopsies were also incubated with the three cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added in combination (3C). Paired biopsies incubated with the three cytokines were also incubated with IL-4, IL-10 or IL-13 at a concentration of 30ng/ml for two hours prior to addition of the cytokines. IL-8 levels were determined in supernatants, using an ELISA specific to IL-8. To correct for differing sizes of biopsies, the protein content of the biopsies was estimated using a protein assay. IL-8 levels are expressed as ng/ml in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of four experiments. A significant effect was found using this dose of both IL-4 and IL-10 compared to samples stimulated with the 3C ($p<0.05$). There was no significant difference between IL-13 and the cytokine stimulated biopsies studied. There was no significant difference between IL-4 and IL-10.

4.2 Discussion And Technical Points

It has previously been shown that the colonic epithelial cells HT-29 are able to produce IL-8 and NO in large quantities when stimulated with the combination of IL-1, TNF-α and IFN-γ. Dr Kolios had previously shown that this production is easily suppressed in a dose dependant fashion using either IL-4 or IL-13 but is resistant to inhibition with IL-10 (Kolios *et al.*, 1998). As the evidence to date suggests that both IL-8 and NO are important in the pathophysiology of inflammatory bowel disease and as we know that IL-10 has been used with some success to treat IBD, there were several possibilities as to why there was a difference. Firstly, was NO or IL-8 production by colonic epithelial cells unaffected by IL-10? Secondly, was the method of stimulating HT-29 cells with IL-1α, IFN-γ and TNF-α to produce NO and IL-8, effectively resistant to suppression with IL-10? Thirdly, was there another cell type that acted directly upon the colonic epithelial cell that was sensitive to IL-10? Lastly, was the HT-29 cell so different to the normal colonic epithelial cell that it was immune to the effect of IL-10?

An experiment was therefore designed to look at inflamed bowel from patients with colitis and examine the effect of incubating them with the three cytokines under investigation. As can be seen from the results, IL-10 does indeed have a marked effect on both IL-8 and NO production as measured by nitrite production. As we know that NO production in the inflamed colon is almost exclusively from colonic epithelial cells this would suggest that IL-10 does indeed act upon colonic epithelial cells or another cell type that directly controls what the epithelial cells do. This would suggest that IL-10 when used in the management of IBD, reduces inflammation at least in part, by inhibiting IL-8 and NO production. Interestingly

neither IL-4 nor IL-13 at the doses examined showed any effect on NO production in this model. IL-4 did have an effect on IL-8 production but there was no effect observed with IL-13. As it is known that both of these cytokines have a marked effect in the epithelial cell line model it suggested that either the conditions used to induce NO production i.e. the combination of IFN-γ, IL-1 and TNF-α in the epithelial cells made them sensitive to IL-4 and IL-13 and resistant to IL-10. The other possibility is that IL-4 and IL-13 were unable to influence sufficiently any other cells acting directly on the epithelial cells. Again in this study compared with other studies, the colonic biopsies are taken from patients who are newly diagnosed colitic patients and are therefore on no treatment prior to the experiment. Other studies looking at immunohistochemistry and other changes in NO biochemistry have used patients who are already well established on treatment often including steroids or commonly on resection specimens from patients who are steroid resistant, thus almost including patients who may have different expression of cytokines and sensitivity of immune cells to cytokines.

This effect of the IL-10 and IL-4 was also confirmed by the data from the PCR results looking at iNOS and IL-8 mRNA. This shows that iNOS is produced in colitis and that the levels of the mRNA are reduced when the inflamed biopsies were incubated with either IL-10 or IL-4. Although the method used is only semi-quantitative the evidence combined with the nitrite and IL-8 data from the incubated biopsies from inflamed colonic biopsies would strongly suggest that this is a real reduction in iNOS and IL-8 production by down regulation of mRNA. It also confirms that IL-13 seems to have no effect on levels of mRNA. Again as in chapter 3, ideally northern blot techniques would have been used, and again this was tried on several occasions. Unfortunately, even after 20 hours of

incubation too much degradation of the mRNA had occurred and this did not allow me to proceed with this method of examining how much mRNA was present. Results from Western blot analysis would have also been presented to confirm a lowering of the product of the iNOS gene. Unfortunately within the time restraints allowed to do the work in, I was unable to do this.

To examine this difference between the biopsies and the HT-29 cells further the stimulated biopsy models were set up. This allowed us to examine the effect of stimulation with the cytokine mixture on the biopsies and to examine whether this created conditions where IL-10 was unable to act. This showed that normal colonic mucosa could produce large quantities of both IL-8 and NO when exposed to IL-1, TNFα and IFN-γ. In both colitic and non-colitic patients, nitrite levels are easily suppressed by both IL-4 or IL-10 suggesting that the cytokine stimulated mucosa behaves as the inflamed mucosa does when examining NO production. The variable effect of IL-13 is harder to explain especially as it is closely related to IL-4 and is thought to use the same cellular receptor. In histologically normal colonic mucosa from patients without colitis, IL-13 significantly inhibits nitrite production, but appears to have no effect on nitrite production from cytokine stimulated normal colonic mucosa from subjects with colitis. This lack of effect of IL-13 mirrors that seen in the inflamed colitic mucosa biopsy model. Why there should be this difference is unclear, but does suggest that even in histologically normal mucosa there are differences that are present in colitic individuals. Whether the structural cells of the colon e.g. epithelial cells, lamina propria cells are abnormal in colitic patients and behave like cells from inflamed mucosa when stimulated in the appropriate manner or whether the more mobile population of

circulating white cells can, when appropriately stimulated, induce a colitis thus explaining the similarities between non-inflamed and inflamed colitic bowel.

When looking at IL-8 production we have shown that normal colonic mucosa both from colitic and non-colitic patients can be stimulated to produce IL-8 in large quantities when incubated with the combination of IL-1, TNF-α and IFN-γ. Pre-treatment with either IL-4 or IL-10 can effectively inhibit this production. However, IL-13 again seems to have no effect on either IL-8 production in both the colitic and non-colitic model.

The general lack of effect of IL-13 seems consistent throughout all the colonic biopsy data even though it was being used at doses that had been shown to be very effective in the stimulated HT-29 model. The other striking difference was that IL-10 seemed very effective in the biopsy data even though there was no effect at all in the stimulated HT-29 cell model.

To examine further why IL-10 has a marked effect on NO production in all the biopsy models but has no effect on HT-29 cells it was proposed that in fact it may be acting upon white cells which in turn have an effect on colonic epithelial cells. It is apparent that as the epithelial cells in the colon are thought to be the exclusive source of NO production that IL-10 must be having an effect either directly on epithelial cells or on other cells that have an effect on the colonic epithelial cells.

Chapter 5: RESULTS

The Interaction of Mixed Mononuclear Cells and Colonic Epithelial Cells. Controlling Factors Affecting Inducible Nitric Oxide Expression

5.1 Effect Of Different Stimuli On A Co-Culture Of HT-29 Cells And Mixed Mononuclear Cells.

With the difference between the ability of steroids and IL-10 to inhibit nitric oxide production in cytokine stimulated HT-29 cells and the stimulated normal mucosa, a series of experiments was set up to see if these responses could be reproduced with HT-29 cells being stimulated by cytokine stimulated mixed mononuclear cells.

Initial experiments were performed to see whether HT-29 cells co-cultured with mixed mononuclear cells produced any significant nitrite production. HT-29 cells and mixed mononuclear cells were cultured on their own and also co-cultured together. A variety of different stimuli were added to see whether conditions could be created where stimuli could be added to the co-culture of the two cell populations and excess nitrite produced over and above baseline nitrite production. The same stimuli when added to the isolated populations of cells should result in no nitrite production over and above basal production. A variety of possible stimuli were examined such as the stimuli already known to induce NO production in the HT-29 cells e.g. IL-1α, TNF-α and IFN-γ. Each of these was tried on their own and in combination with each other and another possible stimuli e.g. lipopolysaccharide.

HT-29 Unstimulated	471 ± 25
HT-29+IL-1+TNF+IFN	1068 ± 158
MMCs alone	446 ± 47
HT-29+ MMCs	481 ± 13
HT-29+ MMCs+ IFN	457 ± 69
HT-29+ MMCs+ IL-1	421 ± 49
HT-29+ MMCs+ TNF	511 ± 113
HT-29+ MMCs+ LPS	496 ± 76
HT-29+ MMCs+ LPS+TNF	386 ± 46
HT-29+ MMCs+LPS+IL-1	354 ± 112
HT-29+ MMCs+LPS +IFN	1425 ± 120
HT-29+ LPS+IFN	676 ± 68
MMCs+ LPS+IFN	568 ± 121

Table 13. Nitrite production by cultures of HT-29 cells and MMCs. Effect of incubation with a variety of pro-inflammatory stimuli. All values expressed as pmoles/10^6 cells

Basal nitrite production by unstimulated growth arrested HT-29 cells at 48 hours was 471 ± 25 pmoles/10^6 cells (mean ± SEM). Nitrite production from isolated mixed mononuclear cells was not significantly different at 446 ± 47 pmoles/10^6 cells. When HT-29 cells were co-cultured with MMCs in control media there was no significant increase in nitrite production over unstimulated HT-29 cells with production at 481 ±13 pmoles/10^6 cells. HT-29 cells stimulated with IFN-γ and LPS did not change nitrite production significantly compared with unstimulated HT-29 cells with production being 676 ±68 pmoles/10^6 cells. Nitrite production in MMCs stimulated with IFN-γ and LPS also did not change significantly with production at 568 ± 120 pmoles/10^6 cells. When co-cultured together and

stimulated with LPS and IFN-γ rose significantly to 1425 ±120 pmoles/10^6 cells ($p<0.05$). (Table 13) (Figure 28). MMCs stimulated with LPS and IFN-γ for 6 hours were then centrifuged at high speed and then the supernatant removed from above the cellular pellet. This conditioned media was then transferred to confluent growth arrested confluent HT-29 cells and incubated for a further 42 hours. This conditioned media was able to cause a significant rise in nitrite production by HT-29 cells at 48 hours to 1380 ± 170 pmoles/10^6 cells ($p<0.05$). (Figure 29)

5.2 Effect Of Prednisolone and Budesonide On A Co-Culture Of HT-29 Cells And Stimulated Mixed Mononuclear Cells.

As stated above it was shown that the combination of LPS and IFN-γ could not induce NO production as measured by an increase in nitrite above basal production in either the HT-29 cells or the MMCs when cultured alone. When the two types of cell were co-cultured together in the presence of LPS and IFN-γ a significant increase in nitrite could be measured. A dose response curve was able to be demonstrated when the co-culture of HT-29 cells and MMCs was pre-treated with either prednisolone or budesonide. A significant suppression occurred at doses of 30nM for prednisolone and all doses of budesonide tested. In a treated co-culture the nitrite production rose from 473 ± 107 pmoles/10^6 cells to 1203 ± 138 pmoles/10^6 cells. The nitrite production at a dose of 30nM fell to 728 ±99 pmoles/10^6 cells for prednisolone ($p<0.05$) and 550 ± 71 pmoles/10^6 cells for budesonide treated cells ($p<0.05$) (Figure 30) (Table 14).

Media transferred from the stimulated MMCs was able to stimulate nitrite production in the HT-29 cells from the basal level of 478 ± 107 pmoles/10^6 cells to 1680 ± 170 pmoles/10^6 cells. A culture of growth arrested HT-29 cells was pre-

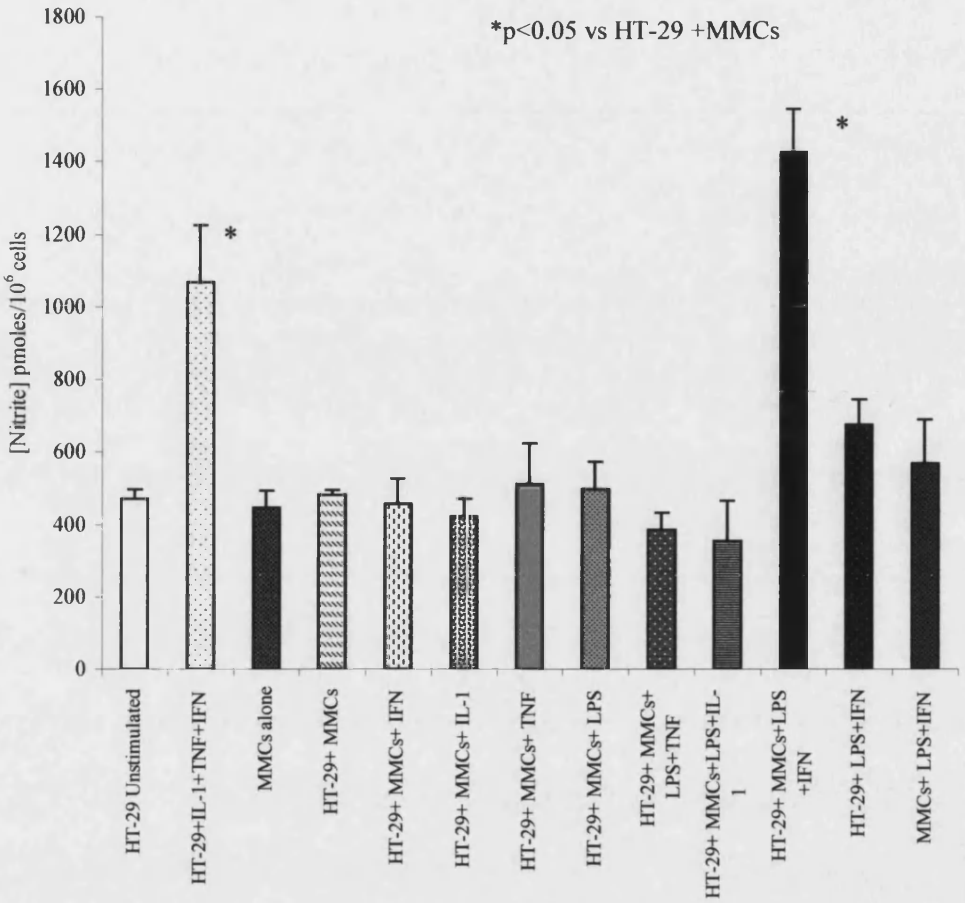


Figure 28. Nitrite production by HT-29 cells and mixed mononuclear cells after 48 hours cultured alone or co-cultured together and stimulated with a variety of different stimuli. Adherent starved HT-29 cells were confluent at the bottom of a standard six well plate at an approximate density of 3.5 x 10^6 cells per well. The MMCs were at a concentration of 500,000/ml. The HT-29 and MMCs when co-cultured with LPS and IFN-γ produced a significant increase in nitrite compared to the co-culture alone.

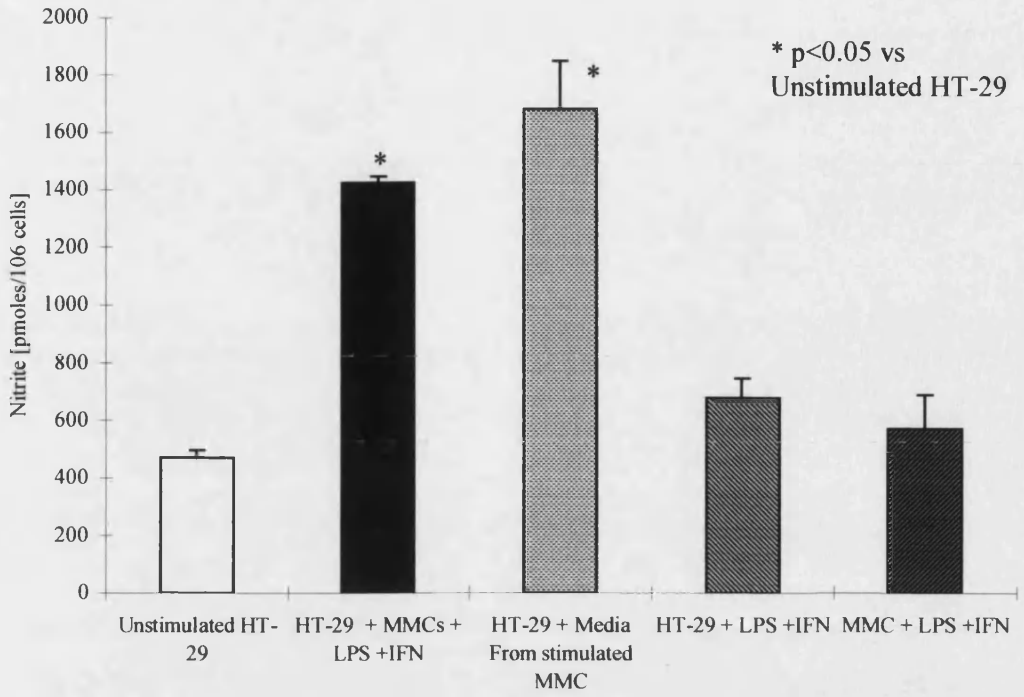


Figure 29. Nitrite production by the colonic epithelial cell line HT-29 and mixed mononuclear cells (MMCs) from normal volunteers (n=6). HT-29 cells and MMCs were incubated at 37°C either separately or together for 30 hours and were stimulated with lipopolysaccharide and IFN-γ (300U/ml) added in combination. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. Nitrite levels are expressed as pmoles in the supernatant per 100μgrams of protein. Each bar is the mean ± SEM of 6 different experiments. The basal production of nitrite by HT-29 cells is shown in the first column. The second column shows nitrite production when MMCs and HT-29 cells when co-cultured together. A significant increase in nitrite production was found when treating the co-culture with the LPS and IFN-γ compared to basal production ($p<0.05$). A significant increase in nitrite production was also found when incubating the HT-29 with media from the LPS and IFN-γ stimulated MMCs compared to basal production ($p<0.05$). No significant increase in nitrite was noted when HT-29 cells were treated with LPS and IFN-γ.

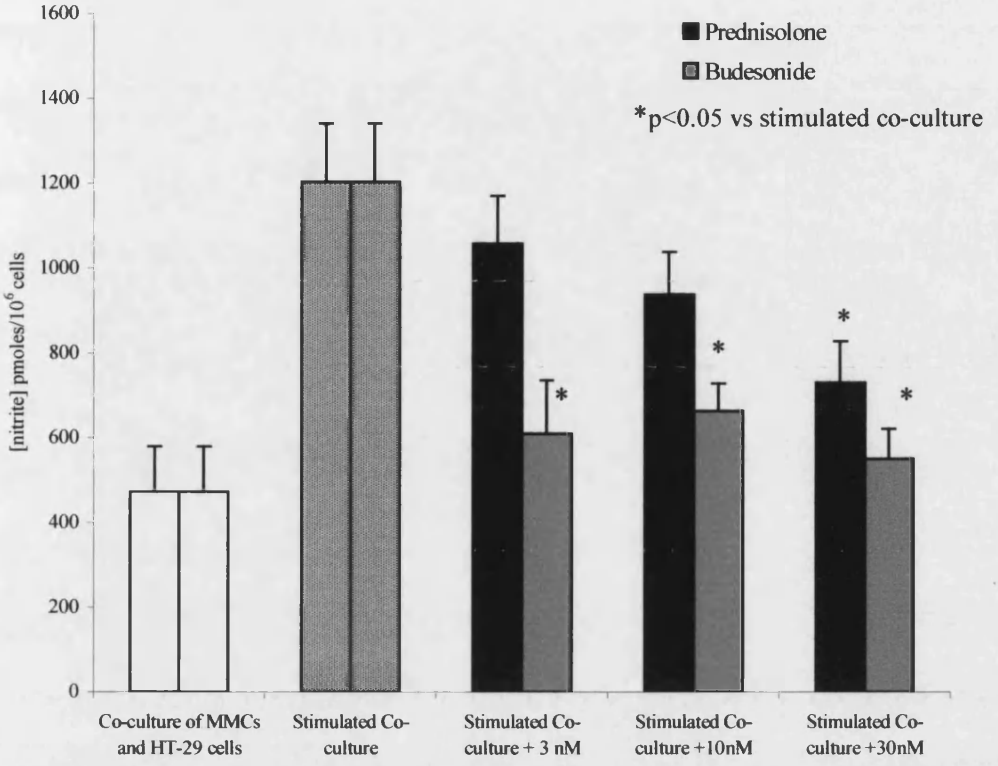


Figure 30. Concentration of nitrite produced by a co-culture of HT-29 cells and MMCs. Adherent starved HT-29 cells were confluent at the bottom of a standard six well plate at an approximate density of 3.5 x 10^6 cells per well. The MMCs were at a concentration of 500,000/ml. The co-cultures were pre-treated with prednisolone or budesonide at one of three different doses for two hours prior to stimulation with LPS and IFN-γ. Supernatant was removed at 48 hours and analysed for nitrite concentration. All three doses of budesonide produced a significant reduction in nitrite generation ($p<0.05$). Prednisolone at a dose of 30nM produced a significant reduction in nitrite generation ($p<0.05$)

treated with either prednisolone or budesonide at a dose of 30nM and then after one hour media from stimulated MMCs was transferred. No significant change was found with either prednisolone or budesonide in nitrite production. With prednisolone this was found to be 1595 ± 126 pmoles/10^6 cells and with budesonide 1486 ± 168 pmoles/10^6 cells. (Figure 31) However, if the MMCs were pre-treated with either prednisolone or budesonide prior at a dose of 30nM prior to stimulation with IFN-γ and LPS and then the conditioned media was transferred then significant falls were found in nitrite production by the HT-29 cells. With prednisolone, nitrite production fell to 624 ± 141 pmoles/10^6 cells ($p<0.05$). With budesonide it fell to 741 ± 91 pmoles/10^6 cells ($p<0.05$). (Figure 32).

	Prednisolone	Budesonide
	473	473
Stimulated Co-culture	1203	1203
Stimulated Co-culture + 3 nM	1057	610
Stimulated Co-culture +10nM	937	663
Stimulated Co-culture +30nM	729	550

Table 14. Nitrite production by cultures of HT-29 cells and MMCs. Effect of incubation with 3, 10 or 30nM of either prednisolone or budesonide. All values expressed as pmoles/10^6 cells

5.3 Effect Of IL-4, IL-10 and IL-13 On A Co-Culture Of HT-29 Cells And Stimulated Mixed Mononuclear Cells.

At 48 hours basal nitrite production by unstimulated HT-29 cells was 478 ± 107 pmoles/10^6 cells (control ± SEM). When the co-culture was stimulated with IFN-γ and LPS nitrite production rose significantly to 1203 ±138 pmoles/10^6 cells ($p<0.05$). The co-culture was pre-treated with IL-4, IL-10 or IL-13 at doses of 3nM, 10nM or 30nM. The cells were then stimulated with LPS and IFN-γ at the doses previously described. A dose response curve for inhibition of nitrite

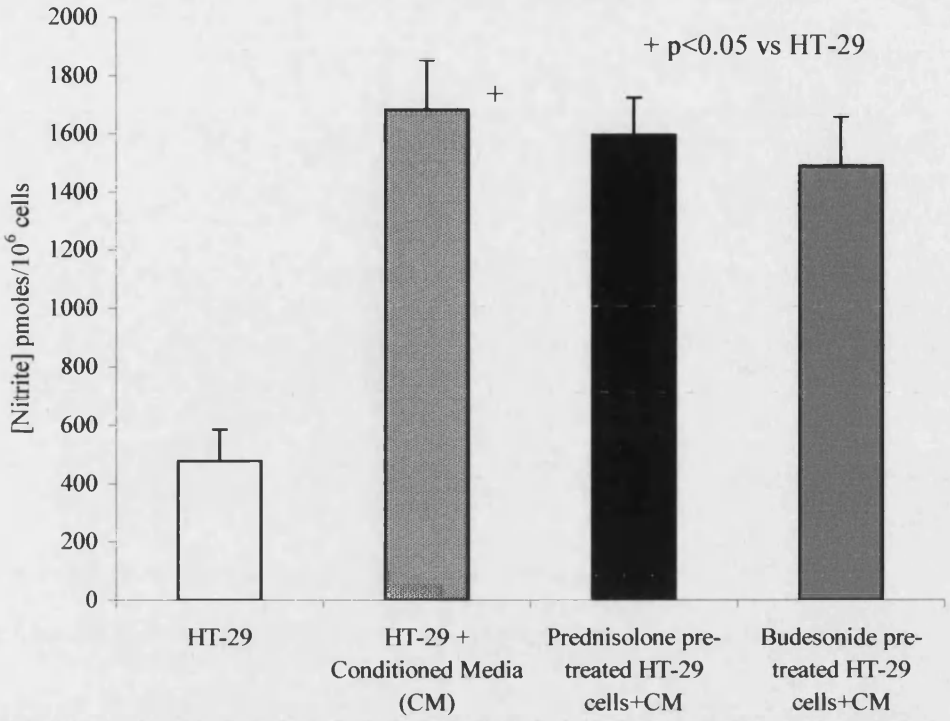


Figure 31. Nitrite production by a culture of serum depleted HT-29 cells treated with conditioned media from MMCs stimulated with LPS and IFN-γ. The effect of pre-treatment with steroids on the HT-29 cells prior to the addition of the conditioned media was assessed. A significant rise in nitrite was found with the addition of the conditioned media. Pre-treatment with prednisolone (30nM) or budesonide (30nM) had no significant effect on the effect of conditioned media.

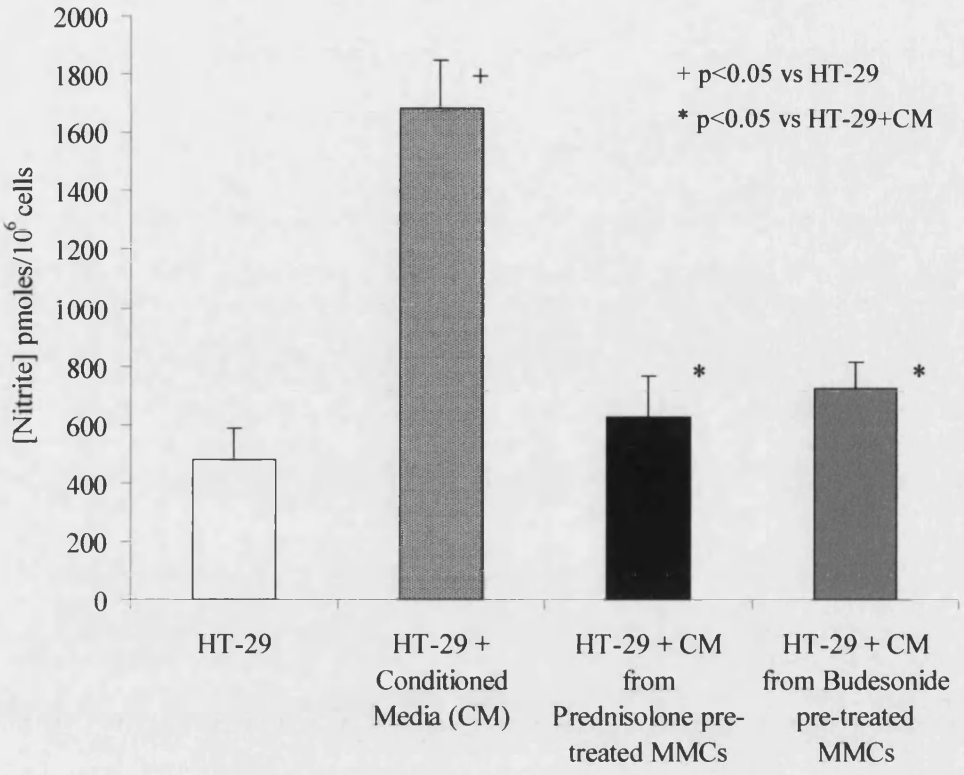


Figure 32. Nitrite production by serum depleted HT-29 cells treated with conditioned media from LPS and IFN-γ stimulated MMCs. The MMCs were pre-treated with prednisolone or budesonide at a concentration of 30nM for 2 hours prior to stimulation with the LPS and IFN-γ. There was a significant decrease in nitrite production by the HT-29 cells when conditioned media was used from MMCs that had been pre-treated with either prednisolone or budesonide ($p<0.05$).

production was demonstrated for pre-treatment with IL-4, IL-10 and IL-13 with a significant suppression for all 3 cytokines at doses of 30nM. In addition pre-treatment with all doses of IL-10 showed a significant fall in nitrite production and IL-4 also showed a significant suppression of nitrite production at 10nM. (Figure 33) (Table 15)

	IL-4	IL-10	IL-13
Co-culture of MMCs and HT-29 cells	478 ± 107	478 ± 107	478 ± 107
Stimulated Co-culture	1203 ± 103	1203 ± 103	1203 ± 103
Stimulated Co-culture + 3 ng/ml	818 ± 103	583 ± 31	1126 ± 58
Stimulated Co-culture +10ng/ml	505 ± 35	468 ± 38	950 ± 102
Stimulated Co-culture +30ng/ml	408 ±41	344 ± 27	666 ± 61

Table 15. Nitrite production by cultures of HT-29 cells and MMCs. Effect of incubation with doses of 3, 10 or 30ng/ml of IL-4, IL-10 or IL-13. All values expressed as pmoles/10^6 cells (mean ± SEM)

Media transferred from the stimulated MMCs was able to stimulate nitrite production in the HT-29 cells from the basal level of 478 ± 107 pmoles/10^6 cells to 1680 ± 170 pmoles/10^6 cells.

	IL-4	IL-10	IL-13
Co-culture Of MMCs and HT-29 cells	478 ± 107	478 ± 107	478 ± 107
HT-29 cells + conditioned media from MMCs	1680 ± 170	1680 ± 170	1680 ± 170
Pre-treated HT-29 cells + conditioned media from MMCs	1775 ± 156	1763 ± 94	1319 ± 157
HT-29 cells + conditioned media from Pre-treated MMCs	818 ± 60	673 ± 76	843 ± 29

Table 16. Nitrite production by cultures of HT-29 cells and MMCs. Effect of pre-treatment of either HT-29 cells or MMCs with doses of 30ng/ml of IL-4, IL-10 or IL-13. All values expressed as pmoles/10^6 cells (mean ± SEM)

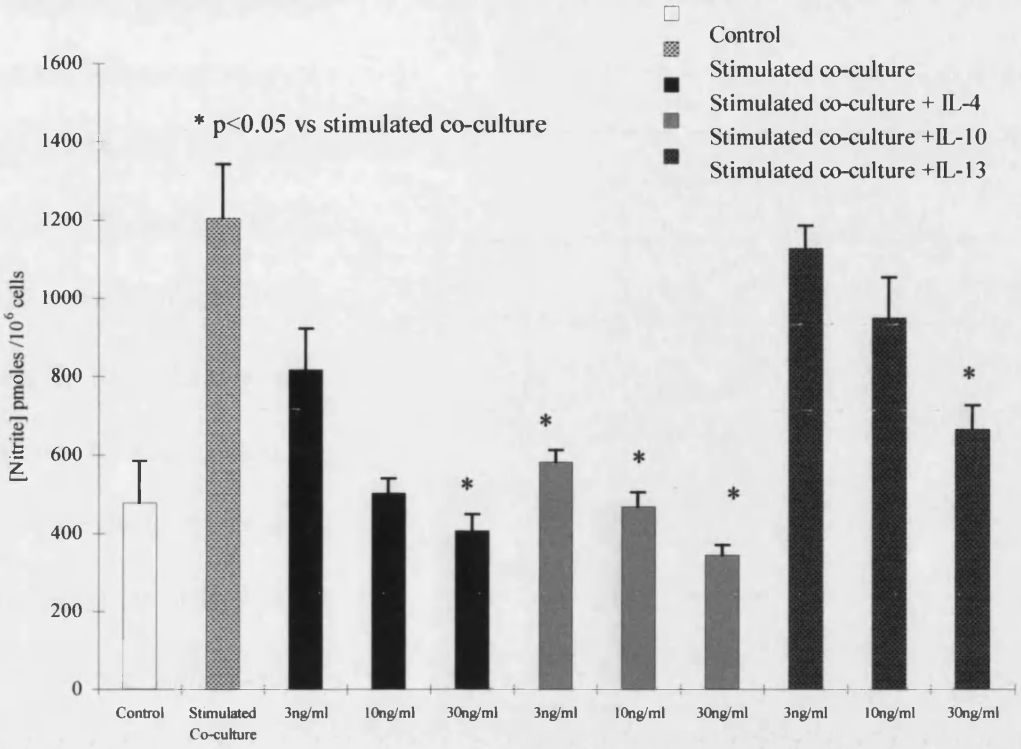


Figure 33. Nitrite concentration in stimulated co-cultures of HT-29 cells and MMCs. The co-cultures were stimulated with LPS and IFN-γ. Prior to stimulation the co-cultures were pre-treated with increasing concentrations of IL-4, IL-10 or IL-13. Three different doses were used of these cytokines: 3,10 and 30 ng/ml. A significant reduction in nitrite was seen at all doses of IL-10, IL-4 and IL-13 had a significant reduction of nitrite at a dose of 30ng/ml. In addition a dose of 10ng/ml of IL-4 was found to reduce nitrite production significantly.

A culture of growth arrested HT-29 cells was pre-treated with either IL-4 IL-10 or IL-13 at a dose of 30ng/ml and then after one hour media from stimulated MMCs was transferred. No significant change in nitrite production was found with IL-4 (1775 ± 156 pmoles/10^6 cells), IL-10 (1763 ± 94 pmoles/10^6 cells) or IL-13 (1319 ±157 pmoles/10^6 cells) (Figure 34). However, if the MMCs were pre-treated with IL-4, IL-10 or IL-13 at a dose of 30ng/ml prior to stimulation with IFN-γ and LPS and the transfer of the conditioned media then significant falls were found in nitrite production by the HT-29 cells. With IL-4, nitrite production fell to 818 ± 60 pmoles/10^6 cells ($p<0.05$). With IL-10 it fell to 673 ± 76 pmoles/10^6 cells ($p<0.05$) and with IL-13 it fell to 843 ±29 pmoles/10^6 cells ($p<0.05$). (Table 16) (Figure 35)

5.4 Effect Of An Inhibitory Substance On A Co-Culture Of HT-29 Cells And Stimulated Mixed Mononuclear Cells.

The effect of the inhibitory substance IL-1 receptor antagonist (IL-1ra) was examined on the co-culture of HT-29 cells and MMCs. Firstly, a series of experiments were performed to find the appropriate dose of the IL-1ra. A growth arrested monolayer of HT-29 cells were pre-treated with a variety of doses of either the IL-1ra and were then stimulated with IL-1α, TNFα and IFN-γ at the doses previously described. When the pre-treated HT-29 cells showed a significant fall in nitrite production and led to similar levels to that produced with only two pro-inflammatory cytokines being used, this dose was then used in future experiments. This dose of either IL-1ra was then used either to pre-treat the stimulated MMCs prior to the addition of the LPS and IFN-γ, or to the HT-29

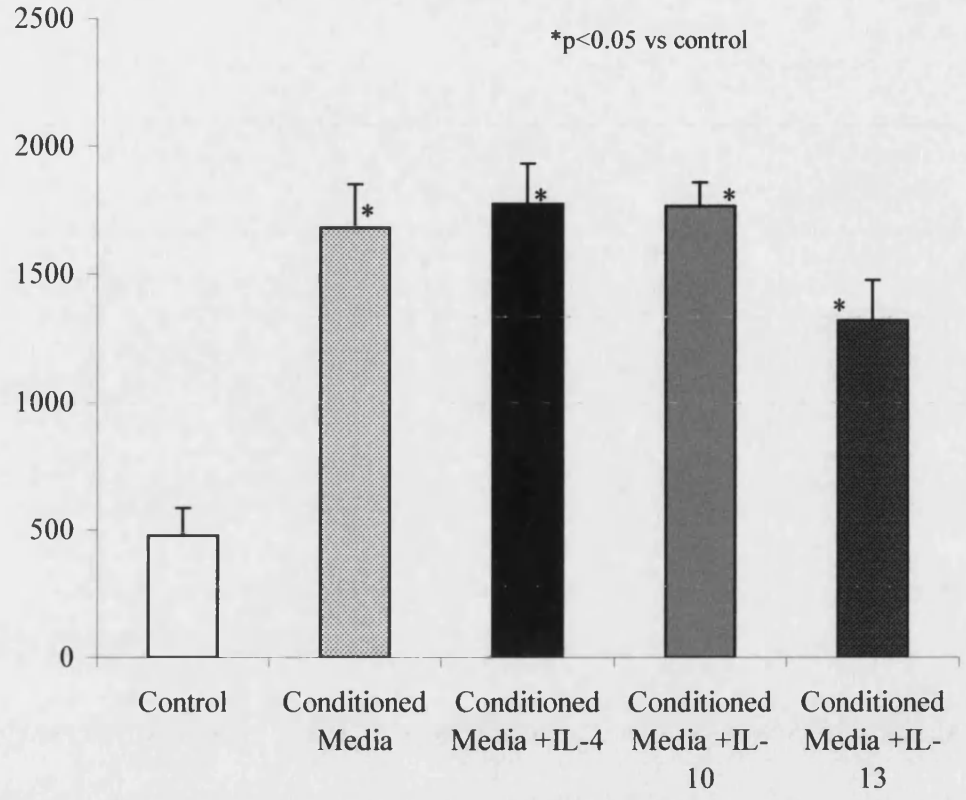


Figure 34. Nitrite production by a culture of serum depleted HT-29 cells treated with conditioned media from MMCs stimulated with LPS and IFN-γ. The effect of pre-treatment with T cell derived cytokines (IL-4, IL-10 and IL-13) on the HT-29 cells prior to the addition of the conditioned media was assessed. A significant rise in nitrite was found with the addition of the conditioned media. Pre-treatment with any of the cytokines had no significant effect on the effect of conditioned media.

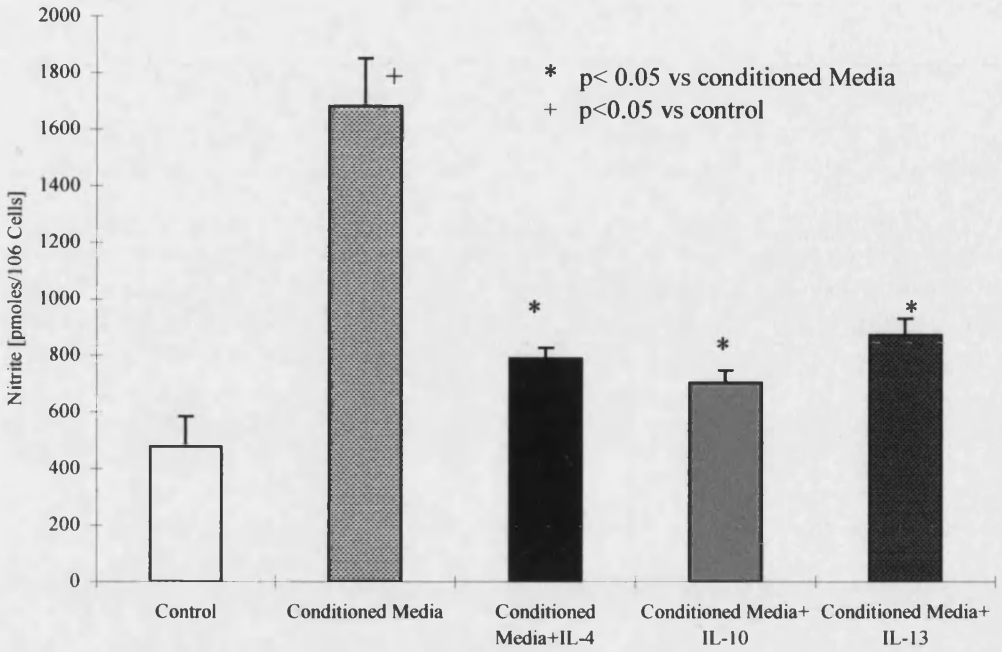


Figure 35 Nitrite production by a culture of serum depleted HT-29 cells treated with conditioned media from MMCs stimulated with LPS and IFN-γ. The effect of pre-treatment with T cell derived cytokines (IL-4, IL-10 and IL-13) on the MMCs cells prior to the addition of the LPS and IFN-γ was assessed. A significant rise in nitrite was found with the addition of the conditioned media. A significant effect of all three of the cytokines on the MMCs was found ($p<0.05$).

cells prior to the addition of the conditioned media. When the HT-29 cells were pre-treated with IL-1ra then nitrite production fell to 991 ± 125 pmoles/10^6 cells ($p<0.05$). If the MMCs were pre-treated with Il-1ra then there was no significant change in nitrite production with levels of nitrite of 1782 ± 151 pmoles/10^6 cells. (Figure 36) ($p<0.05$).

	IL-1ra
Co-culture Of MMCs and HT-29 cells	478 ± 107
HT-29 cells + conditioned media from MMCs	1680 ± 170
Pre-treated HT-29 cells + conditioned media from MMCs	991 ± 125
HT-29 cells + conditioned media from Pre-treated MMCs	1782 ± 151

Table 16. Nitrite production by cultures of HT-29 cells and MMCs. Effect of pre-treatment of either HT-29 cells or MMCs with doses of 30ng/ml of IL-1ra. All values expressed as pmoles/10^6 cells (mean ± SEM)

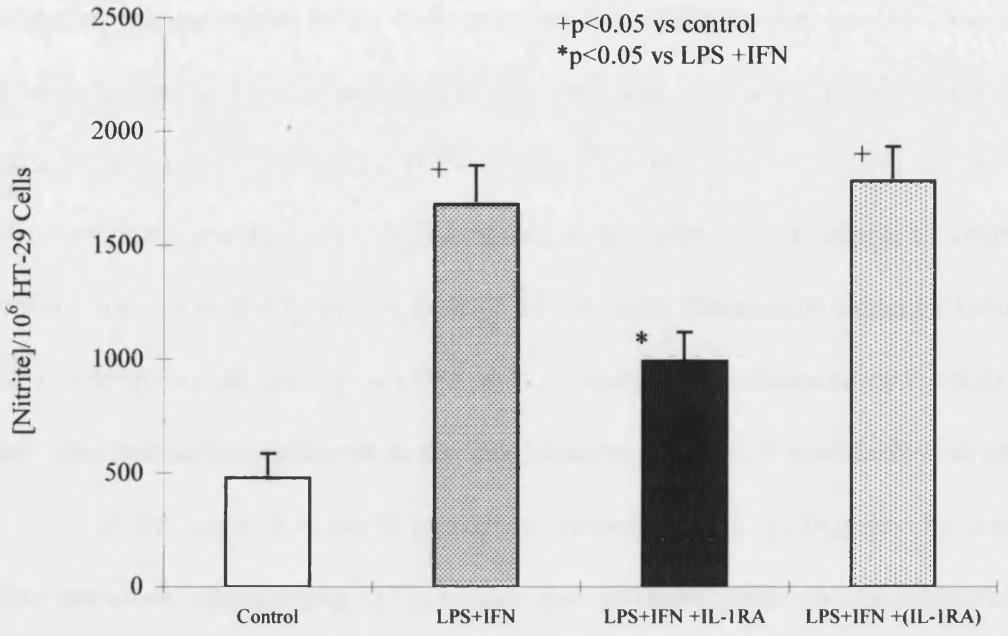


Figure 36 Nitrite production by a culture of serum depleted HT-29 cells treated with conditioned media (CM) from MMCs stimulated with LPS and IFN-γ (n=4). The effect of pre-treatment with IL-1ra on the cells prior to addition of CM was assessed. The effect of pre-treating the MMCs with IL-1ra prior to stimulation with LPS and IFN-γ was also assessed. When the HT-29 cells were pre-treated with the IL-1ra prior to the addition of the CM a significant fall in nitrite was found ($p<0.05$). No change in nitrite production was found when MMCs were pre-treated prior to stimulation with the LPS and IFN-γ.

5.5 Discussion and Technical Points

With the data presented in the early chapters it is apparent that the HT-29 cells behave differently from both inflamed and stimulated biopsies. This difference is most pronounced when looking at the effect of IL-10 on stimulated HT-29 cells. The data first presented by Dr Kolios shows clearly that IL-10 is unable to inhibit NO production by iNOS in a culture of HT-29 cells. Because in inflamed tissue the epithelial cells are not in isolation and are likely to be influenced by cytokines and other substances produced in the inflammatory reaction, it was decided to see if a co-culture could more easily reproduce the results from the biopsies. Because intra-epithelial lymphocytes (IEL) are the likeliest cells to be involved, mononuclear cells were chosen for the co-culture. Although peripheral mixed mononuclear cells are likely to be different in some respects from IELs, they are the easiest population of similar cells to obtain in large enough numbers to perform multiple co-culture experiments.

The author has shown that HT-29 cells stimulated with LPS and IFN-γ do not produce nitrite and that isolated MMCs stimulated with LPS and IFN-γ do not produce excess NO as measured by nitrite. Also when co-cultured together in the absence of any stimulation again no increase in NO production is found. However when HT-29 cells are co-cultured with stimulated MMCs they do produce large quantities of NO. Pre-treating the MMCs with low doses of prednisolone or budesonide easily inhibits this. These doses of steroids are similar to that used in the biopsy models and do not have to be used at the very high doses that have to be used to obtain an effect on the stimulated HT-29 cells.

The data also shows that the HT-29 cells and the MMCs do not need to be in contact and NO production by HT-29 cells can be induced by transferring media

from IFN-γ and LPS stimulated MMCs. This would suggest that although the epithelial cells are the site of production of NO by iNOS, this production is always dependant on stimulation of the epithelial cells with external cellular factors. The fact that media transferred from stimulated MMCs can induce this production shows that these factors are soluble.

This data strongly suggests that the mononuclear cells are the main site of action of the steroids prednisolone and budesonide when considering the reduction in NO production noted in this biopsy study and is the likely cellular site of action in previous studies noting a fall in NO production in treated colitis.

This NO production by the HT-29 cells, as measured by nitrite, is easily inhibited by pre-treating the MMCs with small doses of either IL-4 or IL-10. IL-13 did have an effect but this was much less marked and only at a dose of 30ng/ml. If HT-29 cells were pre-treated with 30ng/ml of IL-4, IL-10 or IL-13 and then transferred conditioned media from the MMCs then no reduction in nitrite was seen. However this ability of the conditioned media to induce nitrite was easily blocked by pre-treating the MMCs with any of the anti-inflammatory cytokines. This data suggests that the production of soluble factor or factors produced by the mononuclear cells can be inhibited by both IL-4, IL-10 and to a lesser extent IL-13. This may explain the effect seen in NO production noted in this biopsy study compared with just the HT-29 cell model when examining IL-10.

Pre-treatment of the HT-29 cells with IL-1ra inhibits the effect of the conditioned media. The effect of the IL-1ra was not seen when directly treating the MMCs with the IL-1ra. This would suggest that IL-1 is one of the soluble factors secreted by the stimulated MMCs that are necessary to induce NO production in colonic epithelial cells.

No data concerning the MMCs and HT-29 model with respect to the production of IL-8 has been presented as when the MMCs are stimulated with IFN-γ and LPS, large quantities of IL-8 are released, thus rendering it impossible to examine the effect on the HT-29 cells.

It has been shown in previous studies that the when HT-29 cells are incubated in the presence of IFN-γ, the cells can become antigen presenting cells. This in theory could suggest that the MMCs are interacting directly with the HT-29 cells once they have in turn become antigen-presenting cells and produce cytokine production and cell death. This would appear unlikely in our study partly because of the small amount of time the two populations of cells are together, and also because of the experiments where the MMCs and the HT-29 cells were cultured apart but that conditioned media from the MMCs was transferred from to the HT-29 cells. This last experiment clearly shows that the two populations of cells do not actually need to be in physical contact but that media removed from stimulated MMCs can induce NO production in the HT-29 cells as measured by nitrite.

This data suggests that although the colonic epithelial cell is essential for the production of NO in any colonic inflammation, it is necessary for other cell types to be present i.e. mixed mononuclear cells.

Chapter 6:

DISCUSSION

The role of the epithelial cell in the aetiology and pathogenesis of inflammatory bowel disease remains unclear. There is now a large amount of evidence to suggest that the epithelial cell not only acts as a barrier and as part of the absorptive surface, but also has an important role in the initiation of the inflammatory response. The role of the epithelial cell in the inflammatory response is suggested by the ability of the epithelial cells to present antigens via class II MHC expression (Lowes *et al.*, 1992), express adhesion molecules such as ICAM-1 (Dippold *et al.*, 1993) and produce soluble mediators such as cytokines (Jung *et al.*, 1995).

It is also known that in inflammatory states of the colon e.g. ulcerative colitis, Crohn's disease and infectious colitis in humans that large quantities of NO are produced (Lundberg *et al.*, 1994;Rachmilewitz *et al.*, 1995a). The colonic epithelial cell has been shown to be the exclusive site of production of NO by iNOS in the inflamed colon. Due to generation of toxic breakdown products such as peroxynitrite, excess nitric oxide has been implicated in the inflammatory response. The effect of inhibition of nitric oxide inhibition has been described in an animal model of colitis. This has shown that the severity of the colitis is ameliorated (Neilly *et al.*, 1996). The relevance of this to the colitis seen in humans in the inflammatory bowel disease is still open to discussion. Indeed, evidence from iNOS "knockout" mice has suggested that nitric oxide as produced by iNOS has a role in reducing the inflammation found in inflammatory bowel

disease (McCafferty *et al.*, 1997). Animal models of colitis are usually rat models, where the colon is exposed to a toxic substance e.g. dextran sulphate or TNBS. Whether this is strictly relevant to the inflammation seen in UC and Crohn's disease is still debated, where the inflammation seen in IBD may be an unusual response to an environmental substance or pathogen but could instead be a primary immunological defect.

IL-8 in contrast to nitric oxide produced by iNOS is produced by many cell types for example immune cells, fibroblasts and endothelial cells as well as the epithelial cell (Baggiolini *et al.*, 1994). Thus changes found when looking at the effect of a particular treatment on a colonic biopsy that showed an effect on iNOS would suggest that the epithelial cell is the primary site of action. If an effect was noted on IL-8 but not on iNOS expression then this would suggest that the epithelial cell is not the primary site of action.

There are many published experiments examining the response of colonic epithelial cell lines to various stimuli. Isolated primary human colonic epithelial cells have many problems being kept alive past 48 hours, thus cell lines are used, as they are immortal. This avoids the possible problem that any response seen in primary epithelial cells could be argued to be the response of a "dying" cell. However, work using cell lines can also be argued to be the response of abnormal cells due to the fact that they have large changes to their genetic structure. However, this may be the only way to study cells such as the colonic epithelial cell in isolation. Previous cell line work studying colinic epithelial cells such as the HT-29 cells has demonstrated that they are able to produce large quantities of nitric oxide and IL-8 when appropriately stimulated (Kolios *et al.*, 1995; Salzman *et al.*, 1996).

The experiments described here were designed to examine the relationship between the responses already described in HT-29 cells and the response of the colonic epithelial cell in the colon. Specifically I was examining the controlling mechanisms of NO production by iNOS and IL-8 production. One of the mainstays of treatment of both ulcerative colitis and Crohn's disease are steroids. It would therefore be expected that steroids would have an effect on NO production by iNOS, an enzyme known to be upregulated in inflammation. Similarly, because it is known that IL-10 deficient mice will naturally develop a colitis unless kept in a sterile environment, IL-10 is thought to be a key "anti-inflammatory" cytokine possibly deficient or defective in those who develop colitis. There has been some limited success with therapeutic trials of IL-10 in the management of inflammatory bowel disease (Schreiber *et al.*, 1995b).

In the experiments described here, there are some marked differences in the experiments using growth arrested monolayers of the immortal HT-29 cells, from the more physiological models described. The stimulated HT-29 cells are relatively immune to suppression of iNOS by steroids, although iNOS expression in other cell lines and cell types, both animal and human, do appear more sensitive to suppression by steroids e.g. the lungs (Yates *et al.*, 1995), rat mesangial cells (Pfeilschifter, 1991), Caco-2 cells (Cavicchi and Whittle, 1999), rat peritoneal neutrophils (McCall *et al.*, 1991) and RAW 264.7 cells (Walker *et al.*, 1997).

The results looking at the production of nitric oxide as measured by nitrite in colonic biopsies, which are inflamed due to colitis, are described here. Firstly there is marked expression of NO in the inflamed mucosa causing a large quantity of nitrite to be able to be measured in the culture media. This is easily measured and would appear to be due to the expression of inducible nitric oxide synthase,

an enzyme not normally detectable in the mucosa. Immunohistochemical studies have shown that there is a strong expression of iNOS in inflamed bowel from patients with colitis (Kolios *et al.*, 1998;Singer *et al.*, 1996;Godkin *et al.*, 1996). The experiments described in chapter 3 show that the enzyme transciption appears to be very sensitive to inhibition by steroids. This would correspond with the expected findings clinically. There is no discernible difference between the two steroids although budesonide is thought to be more potent on a molar for molar basis. This may be explained by the fact that both steroids are being used in dosages that have an effect and to demonstrate a difference we would have to have used even smaller doses of the steroids. It is however very obvious that the doses needed to get any response in the HT-29 cells are approximately 1000 times higher than the doses used that obtained a response in the inflamed biopsies. The dose used in the biopsy model would be much closer to a dose likely to be obtained in the tissues when a patient was treated with oral steroids.

Three possible explanations for this difference exist. Firstly that either the HT-29 cells are immune to steroids, whilst the colonic epithelial cells found in colonic mucosal biopsies are sensitive to steroids. Secondly the stimuli needed to induce iNOS expression in HT-29 cells i.e. IL-1α, IFN-γ and TNF-α do not render colonic epithelial cells immune to the effects of the steroids. It is not known from this model whether the steroids are acting directly on the epithelial cells or are acting upon other cells stimulating the epithelial cells.

To examine this further non-inflamed biopsies from histologically normal colon were taken and stimulated with the same cocktail of pro-inflammatory cytokines e.g. IL-1α, IFN-γ and TNF-α. This again enabled large quantities of nitric oxide to be measured as the breakdown product nitrite. It was shown the enzyme iNOS

was expressed in the stimulated biopsies, suggesting that this was contributing at least in part to the production of the nitric oxide, as no PCR product of iNOS was demonstrable in the unstimulated colonic biopsies, but was clearly present in the stimulated biopsies. Steroids were still found to have a marked effect on the production of IL-8 by the biopsies. As IL-8 is known to be expressed and produced by a wide variety of cell types, not only by the epithelial cell, this data suggests that the steroids are possibly acting upon cell types other than the epithelial cell. To examine this further the HT-29 cell and Mixed mononuclear cell model was developed.

When examining the role of IL-10 in the regulation of iNOS expression, there are again differences between the known work and the small amount of data that is available about the possible clinical action of IL-10. Again because IL-10 is thought to be a key cytokine in the pathogenesis of inflammatory bowel disease, it would be imagined that IL-10 would have an effect on iNOS expression.

From the work of Dr Kolios it is known that iNOS expression by HT-29 cells was unable to be inhibited by IL-10 (Kolios *et al.*, 1998). This is in contrast to the effect of IL-10 on iNOS expression in other cell systems (Cunha *et al.*, 1992). In human macrophages IL-10 reduced NO synthesis by down-regulating iNOS at the transcriptional level (Dugas *et al.*, 1998). Similarly in human keratinocytes IL-10 inhibits IgE mediated iNOS induction and thus reduces NO production (Becherel *et al.*, 1995). However, iNOS expression in murine macrophages treated with TNFα and IFN-γ and avian osteoclast-like cells is actually increased when treated with IL-10 (Sunyer *et al.*, 1996;Zidek and Frankova, 1999). However as in HT-29 cells, rat aortic smooth muscle cells would appear more sensitive to the inhibition

of iNOS induction by IL-13 than IL-10 (Ruetten and Thiemermann, 1997). Again several possible explanations exist, firstly there could be a different regulation of iNOS in colonic epithelial cells compared to other cell types, or the HT-29 cells do not have an intact IL-10 receptor complex pathway due to the fact that they are adenocarcinoma cells and thus have significant changes in their genetic structure or lastly that the target cell of IL-10 in colonic mucosa is different. It is known that IL-10 does bind to the surface of HT-29 cells thus implying that the receptor is present on the cell surface but whether the receptor functions normally is unknown (Bourreille *et al,* 1999). Although Dr Kolios showed no discernible effect of IL-10 on stimulated HT-29 cells, there are marked differences found in both iNOS and IL-8 production when the cells are pre-treated with both IL-4 and IL-13 (Kolios *et al.*, 1998).

The experiments I have described here have demonstrated a clear effect of IL-10 on the colonic biopsies. It reduces production of both IL-8 and NO production due to iNOS in the inflamed biopsies from patients with ulcerative colitis. It is also able to inhibit NO production by inhibition of iNOS in cytokine stimulated histologically normal biopsies. A possible explanation for this includes the potential that IL-10 is acting on another cell type which normally acts upon the epithelial cell to stimulate NO production by induction of iNOS. Another possible explanation is that as with the lack of steroid effect on the colonic epithelial cell line, the combination of IL-1, TNF-α and IFN-γ stimulates the production of nitric oxide in a way that prevents inhibition by IL-10, but would enable inhibition by IL-4 and IL-13.

Thus non-inflamed biopsies from histologically normal colon were again taken and stimulated with the same cocktail of pro-inflammatory cytokines e.g. IL-1α, IFN-γ and TNF-α. This again enabled large quantities of nitric oxide to be measured in the supernatant as the breakdown product nitrite. Again the enzyme iNOS could be shown to be expressed in the biopsies, suggesting that this was contributing at least in part to the production of the nitric oxide, as no PCR product of iNOS was demonstrable in the unstimulated colonic biopsies, but was clearly present in the stimulated biopsies. These experiments also showed that this IL-4, IL-10 and IL-13 were able to suppress NO production by iNOS in stimulated biopsies. IL-4 and IL-10 were found to have a marked effect on the production of IL-8 by the stimulated biopsies. IL-13 had no significant effect on the IL-8 production in these stimulated biopsies.

A further section of the study was to look at histologically normal mucosa from those patients with proven colitis. In patients with colitis that there is often a sharp cut off between inflamed areas and non-inflamed areas despite them being exposed to approximately the same intraluminal conditions. The reason for this is unclear and could be due to a variety of different factors. A possible explanation is that in some way the vascular supply is different in the inflamed areas compared to the non-inflamed areas. Another possible explanation could be that the mucosa behaves differently between the two areas to the presence of the stimuli that initites the inflammatory response in colitis. This different response could be initiated by differences in the colonic epithelial cell. Thus the same stimulation experiments of histologically normal mucosa from patients with active colitis were performed as on the normal mucosa from patients whose colonic mucosa was normal throughout. These experiments showed that the NO

production in histologically normal mucosa from colitic patients was low and approximately equal to the NO production from normal mucosa. When stimulated with the pro-inflammatory cytokine mix the NO production rose sharply. This production could be inhibited by either the prednisolone or the budesonide in a similar way to that from the stimulated normal mucosa from patients without colitis. A similar response was also seen when looking at IL-8 production by the biopsies. Again the IL-8 levels were low in non-stimulated biopsies and then rose to high levels when stimulated with the pro-inflammatory cytomix. Incubating the stimulated biopsies with either of the two steroids could inhibit this production of IL-8.

With these differences in responses to both steroids and IL-10 between the inflamed biopsies, the cytokine stimulated histologically normal biopsies and the cytokine stimulated HT-29 cells further experiments were designed to examine if these responses could be reproduced. The basis of this was the model of a co-culture of HT-29 cells and mixed mononuclear cells. The experiments were designed to see if a single stimulus or group of stimuli could be found that led to the mixed mononuclear cells being able to stimulate in turn the HT-29 cells. I have first shown that HT-29 cells treated with LPS and IFN-γ do not produce nitrite above the basal level and that LPS and IFN-γ stimulated MMCs do not produce excess NO. Also when co-cultured together in the absence of any stimulation again no increase in NO production is found. However when HT-29 cells are co-cultured with LPS and IFN-γ stimulated MMCs they do produce large quantities of NO.

Pre-treating the MMCs with low doses of prednisolone or budesonide easily inhibited this production of large quantities of NO. Again the doses that are

needed to be used are 1000 times less than those needed to get any inhibition in directly stimulated HT-29 cells. A further set of experiments were done with the "conditioned" media being transferred from stimulated MMCs again being able to show significant production of NO by the HT-29 cells. This shows that the HT-29 cells and the MMCs do not need to be in contact and NO production by HT-29 cells can be induced by transferring media containing soluble factors produced by the IFN-γ and LPS stimulated MMCs. This can be easily inhibited by low doses of steroids, as is the production by the inflamed biopsies. This suggests that although the epithelial cell is the site of production of NO by iNOS, this production in the mucosa is likely to be dependant on stimulation of the epithelial cells with external cellular factors.

This data considering the reduction in NO production noted in this biopsy study strongly suggests that mononuclear cells are likely to be the main site of action of the steroids prednisolone and budesonide. This is the probable site of action when considering the colon as a whole especially with studies noting a fall in NO production in treated colitis (Lundberg *et al.*, 1994). This may allow the mechanism of this down regulation to be explored further thus allowing a more precise mechanism of treatment in colitis.

I also set out to examine the effect of the three "anti-inflammatory" cytokines, IL-4, IL-10 and IL-13 on this co-culture of stimulated MMCs and HT-29 cells. The production of nitric oxide is easily inhibited by pre-treating the MMCs with small doses of either IL-4 or IL-10 (3ng/ml). IL-13 did have an effect but this was much less marked and only at a dose of 30ng/ml. Again, the soluble factors in the conditioned media from the stimulated MMCs were shown to stimulate the HT-29 cells to produce nitric oxide.

This co-culture model was then used to try and ascertain the principal site of action of the anti-inflammatory cytokines. Because we know that this production can be induced by the conditioned media transferred from stimulated MMCs thus showing that this media contained factor or factors that are soluble. If we pre-treated the HT-29 cells with 30ng/ml of IL-4, IL-10 or IL-13 and then transferred conditioned media from the MMCs then no reduction in nitrite was seen. However this ability of the conditioned media to induce nitrite was easily blocked by pre-treating the MMCs with any of the anti-inflammatory cytokines. This data leads to two conclusions. Firstly it suggests that the mononuclear cells produce a soluble factor that induces colonic epithelial cells to produce nitric oxide. Secondly, that the production of these factors by MMCs are inhibited by both IL-4, IL-10 and to a lesser extent IL-13. This may explain the effect seen in NO production noted in this biopsy study compared with just the HT-29 cell model when examining IL-10. One interesting observation from these experiments with the transfer of conditioned media is that nitric oxide production by the HT-29 cells is not inhibited by pre-treating with the IL-4 or IL-13 unlike in the experiments described by Dr Kolios where HT-29 cells were stimulated directly with IL-1, TNF and IFN-γ. This may suggest that the HT-29 cells are being stimulated into producing nitric oxide with different factors, rather than the IL-1, TNF-α and IFN-γ.

The effect of the conditioned media could also be inhibited by pre-treating the HT-29 cells with IL-1ra. The effect of the IL-1ra was not seen when directly treating the MMCs with the IL-1ra. This would suggest that IL-1 is one of the soluble factors secreted by the stimulated MMCs that are necessary to induce NO production in colonic epithelial cells.

No data concerning the MMCs and HT-29 model with respect to the production of IL-8 has been presented as when LPS and IFN-γ stimulate the MMCs, large quantities of IL-8 are released, thus rendering it impossible to examine the effect of either steroids or the "anti-inflammatory cytokines" on the HT-29 cells.

Despite steroids being used in the treatment of inflammatory bowel disease since the 1950s there is still a lack of understanding of how exactly steroids work. There is a large amount of published data looking at the actions of steroids, showing that they have a wide variety of actions. Glucocorticoid hormones are effective in controlling inflammation, but the mechanisms that confer this action are largely unknown. The genes whose activities are modulated in the anti-inflammatory process code for important components of the inflammatory process such as cytokines, adhesion molecules and enzymes. Most of them do not carry a classical binding site for regulation by a glucocorticoid receptor, but have instead regulatory sequence for transcription factors such as NF-kappa B. It is thought that steroids may inhibit NF-kappa B transcription factor (Barnes and Adcock, 1993; Cato and Wade, 1996) The exact cellular site of action is unknown. If this could be determined then it is possible that new therapies could be designed that are designed to act on a particular cell or cell type perhaps thus enabling control of these chronic diseases without subjecting the patient to the high risk of side effects of steroids. Despite extensive investigation of newer therapies such as IL-10 and an understanding of some of its actions, there is still a large amount unknown both about its mechanism, and possibly more importantly, its site of action.

Again, the exact role of nitric oxide in the chronic inflammatory response of inflammatory bowel disease is unknown, yet it would appear to be important, probably when produced in excess by iNOS. It is known that the colonic epithelial cell is essential for the production of NO in any colonic inflammation, and this research would suggest that it is necessary for other cell types to be present; i.e. mixed mononuclear cells. This may allow more specific targeting of therapeutic agents to reduce NO production and thus inflammation in IBD.

Conclusions

There does appear to be significant differences between the behaviour of the HT-29 cell line model and the colonic epithelial cell in the biopsy model. How much of this is due to differences between an epithelial cell from a cell line and the *in vivo* colonic epithelial cell or to how the epithelial cell line is stimulated in models is still unclear. However, it is likely that a large part of this difference is due to the influence of surrounding cells.

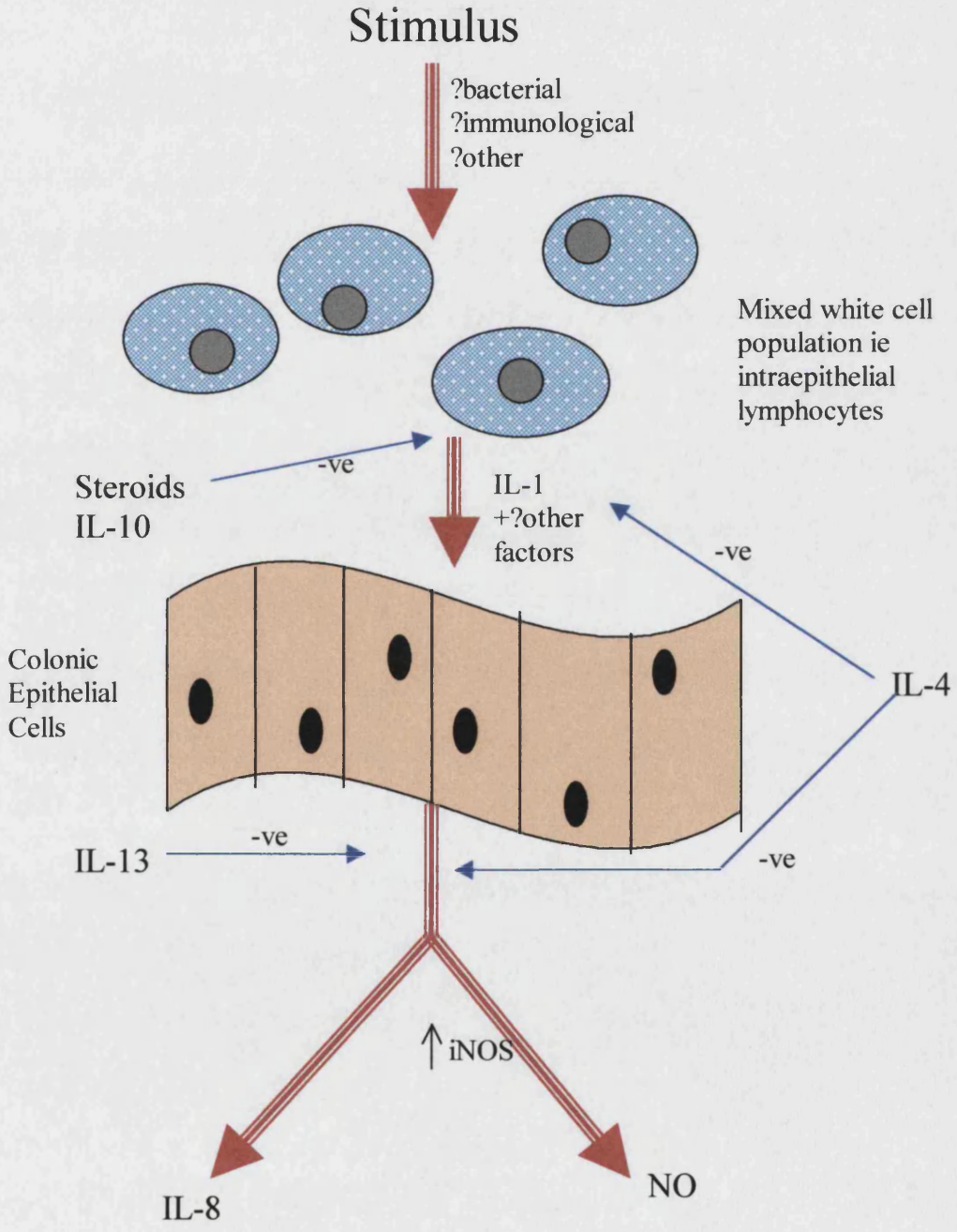
Expression of iNOS in colonic epithelial cells does seem to be sensitive to therapeutic doses of both prednisolone and budesonide, but that these drugs do not appear to act directly upon the epithelial cell itself but upon cells in the surrounding tissues. Any difference found in IBD, is likely to be due to the intraepithelial lymphocytes and other mixed mononuclear cells present in inflamed colonic mucosa.

IL-10, as well as IL-4 and to a lesser extent IL-13, has a powerful effect on colonic epithelial cell as measured by iNOS expression in the biopsy model of IBD in contrast to cell line work published earlier. Again this difference between the two models is likely to be due to an effect of IL-10 on surrounding cells, primarily mixed mononuclear cells.

These surrounding white cells stimulate nitric oxide production by the production of soluble factors, and that one of these factors is almost certainly IL-1, as demonstrated by the ability of IL-1ra to partially inhibit this production.

Proposed Model Of The Role Of IL-4, IL-10,IL-13 And Steroids In

Colonic Mucosal Inflammation.



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